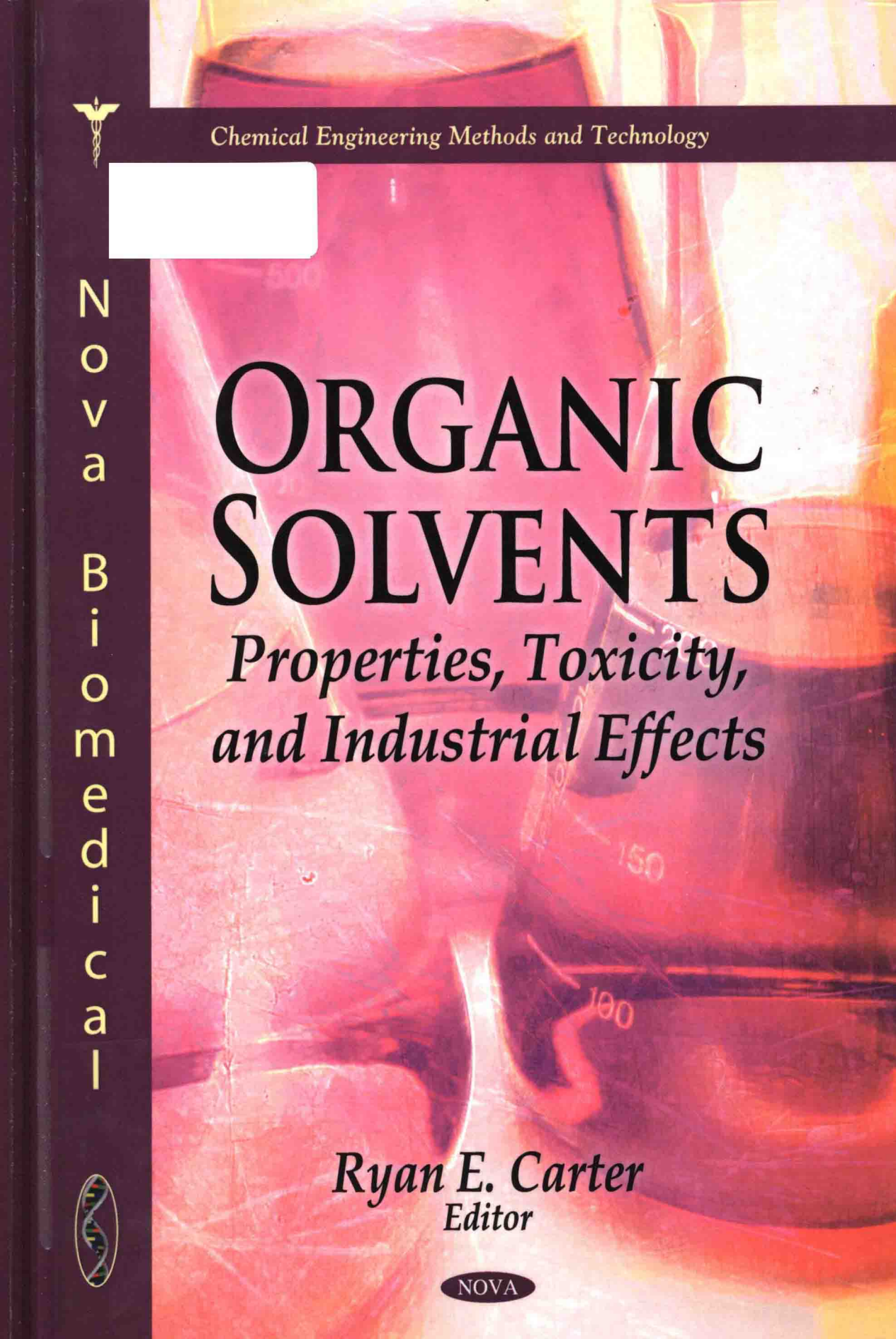

Chemical Engineering Methods and Technology
Nova Biomedical
ORGANIC SOLVENTS
Properties, Toxicity, and Industrial Effects
Ryan E. Carter
Editor
NOVA

CHEMICAL ENGINEERING METHODS AND TECHNOLOGY

ORGANIC SOLVENTS: PROPERTIES, TOXICITY, AND INDUSTRIAL EFFECTS

RYAN E. CARTER
EDITOR

Nova Science Publishers, Inc.
New York

LIBRARY OF CONGRESS CATALOGING-IN-PUBLICATION DATA
Organic solvents : properties, toxicity, and industrial effects / editor,
Ryan E. Carter.
p. cm.
Includes bibliographical references and index.
ISBN 978-1-61761-881-9 (hardcover)
1. Organic solvents. I. Carter, Ryan E.
TP247.5.O74 2010
660'.29482--dc22
2010034018

Published by Nova Science Publishers, Inc. † New York

CHEMICAL ENGINEERING METHODS AND TECHNOLOGY

ORGANIC SOLVENTS: PROPERTIES, TOXICITY, AND INDUSTRIAL EFFECTS

CHEMICAL ENGINEERING METHODS AND TECHNOLOGY

Additional books in this series can be found on Nova's website under the Series tab.

Additional E-books in this series can be found on Nova's website under the E-books tab.

BIOTECHNOLOGY IN AGRICULTURE, INDUSTRY AND MEDICINE

Additional books in this series can be found on Nova's website under the Series tab.

Additional E-books in this series can be found on Nova's website under the E-books tab.

PREFACE

A solvent is a liquid that becomes a solution by dissolving a solid, liquid, or gaseous solute. The most common solvent in everyday life is water. This book presents topical research data in the study of organic solvents, including co-solvent applications for biological systems; studying the structure of dehydrated proteins in the presence of organic solvents; the applications of Gram-positive bacteria in organic solvents; as well as the toxicity of organic solvents and ionic liquids to lactic acid-producing microbes.

Chapter 1 - Biological macromolecules and viruses are used as therapeutic reagents and drugs. These important products are processed and stabilized using co-solvents, which are comprised of amino acids, salts, or organic solvents present at high concentrations. They exert their effects through weak interactions with the surface of biological macromolecules and viruses. Co-solvents can have a wide range of stabilizing, or destabilizing, effects on the solutes (i.e., proteins and viruses), depending not only on the type of co-solvent, but its concentration, as well as the type of solute examined. Furthermore, co-solvents can significantly affect the solubility of the system by either salting-in (solubility enhanced) or salting-out (precipitate) the macromolecules. Application of co-solvent systems to a wide variety of macromolecules as well as their mechanism of action will be reviewed.

Chapter 2 - 6-*O*-Lauroyl saccharides were synthesized through condensation in acetonitrile, acetone and 2-methyl-2-propanol with various water contents using the immobilized lipase from *Candida antarctica*. Glucose, galactose, mannose and fructose were used as a hydrophilic substrate. The apparent equilibrium constant, K_C, based on the concentrations of substrates and products in acetonitrile could be correlated to the dynamic hydration numbers of the saccharides. This indicates that the water activity played an important role during the condensation reaction in the microaqueous water-miscible solvent. The K_C for the synthesis of lauroyl mannose also depended on the kind of solvent and could be correlated with the relative dielectric constant, ε_r, of the organic solvent. Acyl mannoses with the acyl chain lengths of 8 to 16 were continuously produced using a plug-flow-type reactor packed with an immobilized lipase. Irrespective of the acyl chain length, a conversion of more than 0.5 was achieved at the superficial residence time, τ_0, equal to or longer than 20 min. A long-term operational stability of the enzyme was examined, and a conversion of *ca.* 40% was maintained for at least 16 days. The productivity was evaluated to be 350 g/L-reactor·day. The surfactant properties of the produced capryloyl, caproyl, lauroyl and myristoyl mannoses were also determined. Three lauroyl phenolic glycosides were synthesized through the condensation of phenolic glycoside such as arbutin, naringin and

phloridzin with lauric acid by immobilized lipase in various organic solvents. The conversion depended on the polarity of the organic solvent used for the reaction. The suppressive effect of each lauroyl phenolic glycoside against the oxidation of linoleic acid was higher than that of the corresponding phenolic glycoside, whereas there was no difference between the radical scavenging activities of unmodified and lauroyl phenolic glycosides. It is suggested that enhancement of the suppressive effect against lipid oxidation is ascribed to the increase in the hydrophobicity of phenolic glycoside by the acylation.

Chapter 3 - This review describes the basic principles of a novel method for studying the structure of the dehydrated proteins in the presence of organic solvents. This method, based on combined calorimetric and FTIR spectroscopic measurements, allows the simultaneous monitoring of the thermochemical parameters (interaction enthalpies, DSC thermograms) of the dried proteins and the corresponding changes in the protein structure in anhydrous organic solvents.

This review aims to analyse the effect of organic solvents on dehydrated protein systems in order to understand what intra- and intermolecular processes produce the main effect on the structure and functioning of proteins in low water organic media.

Two unrelated proteins with a high α-helix content (human serum albumin, HSA) and with a high β-sheet content (bovine pancreatic α-chymotrypsin, CT) were used as models. Two groups of model organic solvents were used. The first group included hydrogen bond accepting solvents. The second group included hydrogen bond donating liquids.

The results obtained showed that:

1) The enthalpy and integral structural changes accompanying the interaction of dried proteins with anhydrous organic solvents depend cooperatively on the solvent hydrophilicity. The solvent hydrophilicity was characterized by an excess molar Gibbs energy of water in organic solvent at infinite dilution and 25°C. Based on this solvent hydrophilicity parameter, the solvents were divided into two groups. The first group included hydrophilic solvents such as methanol, ethanol, and dimethylsulphoxide (DMSO). Considerable structural rearrangements were observed in this group of solvents. The interaction enthalpies of the dried proteins with hydrophilic liquids were strongly exothermic. The second group included the hydrophobic and medium hydrophilic liquids such as benzene, dioxane, butanol-1, and propanol-1. The enthalpy and structural changes in the second group of solvents were close to zero.
2) The FTIR spectroscopic results can be attributed to the formation of different unfolded states of CT and HSA obtained upon dehydration-, alcohol- and DMSO-induced denaturation. The denatured state obtained in DMSO has a maximal degree of unfolding compared with that observed in alcohols or in the presence of dry air.
3) The effect of the organic solvent on the protein structure is "protein selective". On the other hand, the organic solvent-induced integral structural changes versus solvent hydrophilicity profiles do not depend on the predominant form of secondary structure in the protein.
4) Heat-induced exothermic peaks were observed on the DSC thermograms of the dried proteins in anhydrous organic solvents in the temperature range 60-105 °C. This means that dehydrated proteins in anhydrous solvents is the non-equilibrium state at room temperature. These results give strong support to the

idea that the non-equilibrium status of the dehydrated proteins results from the protein–organic solvent interactions being "frozen" at near room temperature.

The thermodynamic and structural data were analysed to give a unified picture of the state of the dried proteins in anhydrous organic solvents. According to this model, the dehydration-induced protein-protein contacts and the potential of the organic solvent to form the hydrogen bonds are key factors in determining the structure of the dehydrated proteins in the liquids under study.

Chapter 4 - Organic solvents have often a deleterious effect on microbial cells, yet the number of isolated bacteria able to thrive in organic environment has been steadily increasing since the first report of such a particular extremophile in the late 1980s. Solvent tolerant bacteria are particularly appealing for application in biocatalysis, since plenty of relevant educts and products are poorly water soluble. This feature critically limits process productivity in aqueous media, although an opposite picture results if bioconversions are performed in organic environment. Similarly, solvent tolerant bacteria can be of use in bioremediation. Given their potential and relevance, dedicated efforts have been made in order to get a significant insight on the mechanisms underlying solvent tolerance. This is mostly due to cell wall adaptation and rapid repair mechanisms, solvent efflux pumps or enzymatic pathways allowing the mineralization of the deleterious solvent. Although several Gram-positive bacteria (viz. *Rhodococcus* spp., *Mycobacterium* spp.) have been effectively used for biocatalysis and bioremediation in the presence of organic solvents, and the mechanisms for solvent endurance have been looked into, the information most widely disseminated on these matters relies on Gram-negative model systems. The present review aims to provide an updated perspective on the applications of Gram-positive bacteria in organic solvents environment, their mechanisms of tolerance and foreseen developments

Chapter 5 - *In situ* extractive fermentation of lactic acid using organic solvents has already been extensively investigated. Now ionic liquids are emerging as alternative solvents for volatile organic compounds traditionally used in liquid-liquid extraction. We examined whether lactic acid producing-microbes can grow in the presence of a second phase of imidazolium-based ionic liquids or organic solvents. The lactic acid-producing bacteria used in this study are sensitive to organic solvents having $1 < \log P < 4$. Solvent toxicity to lactic acid-producing fungi, *Rhizopus oryzae* JCM 5568, clearly depended on the log *P* values compared with those of the lactic acid-producing bacteria. We found that *Lactobacillus delbruekii subsp. lactis* NRIC 1683 grew in the presence of a second phase of imidazolium-based ionic liquids as well as in the absence of ionic liquids. Finally we discussed the greenness of an ionic liquid system.

Chapter 6 - This review aims to analyse the studies of the competitive inhibitor binding and the storage stability of bovine pancreatic α-chymotrypsin (CT) in organic solvents in order to elucidate what intermolecular processes produce the main effect on the state and functioning of enzymes at high and low water activities in organic media. The binding of competitive inhibitor proflavin and the storage stability of CT in water-organic mixtures were studied in the entire range of thermodynamic water activities (a_w) at 25°C. The moderate-strength hydrogen bond accepting solvents (acetonitrile, dioxane, tetrahydrofuran, and acetone) were used as models due to their ability to vary significantly the size, polarity, denaturation capacity, and hydrophobicity.

The state of water hydrogen bond network in organic solvents was characterized by thermodynamic and spectroscopic data. The absorption spectra of water in organic solvents were measured by FTIR spectroscopy. The state of water in organic solvents was defined in terms of variations in the integral intensity of water and the contour shape of the band of OH stretching vibrations. Excess chemical potentials, partial molar enthalpies, and entropies of water and organic solvents were simultaneously evaluated at 25°C.

The results obtained showed that:

1) The proflavin binding and storage stability curves can be unified in the water activity coordinates. At the highest water activities (a_w>0.95), the water hydrogen bond network is bond –percolated. In this composition region, the storage stability values are close to 100%.
2) At the lowest water activities, the water molecules exist predominantly as single molecules complexed with organic solvent molecules. No proflavin binding was observed at low water activity values in the studied solvents. At a_w>0.3, the proflavin binding is sharply increased reaching a maximal value at a_w~0.5-0.6. This sharp increase in the enzyme activity occurs only above the threshold water activity level, when the self-associated (H-bonded) water molecules appear in the studied organic solvents.
3) In the intermediate composition region, the solution consists of two kinds of clusters, each rich in each component. There is a sharp transition from the water-rich region to the intermediate one. This transition is associated with an anomaly in the thermodynamic, structural, and enzyme activity properties. This transition may involve loss of the bond percolated nature of the hydrogen bond network of liquid water. The residual catalytic activity of CT changes from 100 to 0% in the transition region. A minimum on the competitive inhibitor binding and storage stability curves was observed at a_w of 0.8-0.9.

The thermodynamic, structural, and enzyme activity data were analysed to give a unified picture of the state of enzymes in low water organic solvents. According to this model, the dehydration-induced protein-protein contacts and the state of water hydrogen bond network play a key role in determining the enzyme activity – water activity profiles in organic liquids.

Chapter 7 - The processes of swelling and diffusion of solvents into the structure of tetraflurethylene-propylene coolymer can not be described quantatively using only one characteristic of a solvent. In all cases a molar volume of liquid has the determining effect, hampering its penetration into the structure of polymer. However, the effects of other factors – both solvation and density of cohesion energy of solvents – are significant. Adequate quantitative generalization of abovementioned processes can be obtained on the basis of free energies linearity concept by using of linear multiparameter equations taking into account the effects of different factors.

CONTENTS

Preface		**vii**
Chapter 1	Co-Solvent Application for Biological Systems *Satoshi Ohtake, Yoshiko Kita, Chiaki Nishimura and Tsutomu Arakawa*	**1**
Chapter 2	Lipase-Catalyzed Synthesis of Edible Surfactants in Microaqueous Organic Solvents *Yoshiyuki Watanabe and Shuji Adachi*	**31**
Chapter 3	Analysis of the Organic Solvent Effect on the Structure of Dehydrated Proteins by Isothermal Calorimetry, Differential Scanning Calorimetry and FTIR Spectroscopy *Vladimir A. Sirotkin*	**57**
Chapter 4	Organic-Solvent Tolerant Gram-Positive Bacteria: Applications and Mechanisms of Tolerance *Pedro Fernandes, Marco P.C. Marques, Filipe Carvalho and Carla C.C.R. de Carvalho*	**89**
Chapter 5	Toxicity of Organic Solvents and Ionic Liquids to Lactic Acid-Producing Microbes *Michiaki Matsumoto*	**105**
Chapter 6	Effect of Hydrogen Bond Accepting Organic Solvents on the Binding of Competitive Inhibitor and Storage Stability of α-Chymotrypsin *Vladimir A. Sirotkin*	**115**
Chapter 7	Regularities of Organic Solvents Penetration into Tetrafluoroethylene-Propylene Copolymer *I. Yu Yevchuk, G. G. Midyan, R. G. Makitra, G. E. Zaikov, G. I. Khovanets' and O. Ya. Palchykova*	**153**
Index		**167**

In: Organic Solvents
Editor: Ryan E. Carter

ISBN 978-1-61761-881-9

Chapter 1

CO-SOLVENT APPLICATION FOR BIOLOGICAL SYSTEMS

Satoshi Ohtake[1]*, Yoshiko Kita[2]•, Chiaki Nishimura[3]& and Tsutomu Arakawa[4]♦

1. Aridis Pharmaceuticals, San Jose, CA
2. Department of Pharmacology,
KEIO University School of Medicine, Tokyo, Japan
3. Faculty of Pharmaceutical Sciences,
Teikyo Heisei University, Chiba, Japan
4. Alliance Protein Laboratories, Thousand Oaks, CA

ABSTRACT

Biological macromolecules and viruses are used as therapeutic reagents and drugs. These important products are processed and stabilized using co-solvents, which are comprised of amino acids, salts, or organic solvents present at high concentrations. They exert their effects through weak interactions with the surface of biological macromolecules and viruses. Co-solvents can have a wide range of stabilizing, or destabilizing, effects on the solutes (i.e., proteins and viruses), depending not only on the type of co-solvent, but its concentration, as well as the type of solute examined. Furthermore, co-solvents can significantly affect the solubility of the system by either salting-in (solubility enhanced) or salting-out (precipitate) the macromolecules. Application of co-solvent systems to a wide variety of macromolecules as well as their mechanism of action will be reviewed.

* Address correspondences to Satoshi Ohtake, Aridis Pharmaceuticals, 5941 Optical Court, San Jose, CA 95138.
• 35 Shinanomachi,Shinjuku-ku, Tokyo 160-8582, Japan.
& 4-1 Uruido-minami, Ichihara, Chiba 290-0193, Japan.
♦ 3957 Corte Cancion, Thousand Oaks, CA 91360.

1. INTRODUCTION

Biological macromolecules and viruses are used as therapeutic reagents and drugs for basic research and drug development. Physico-chemical properties of biological macromolecules, such as their stability, solubility, and functional properties, are modulated by solvent composition. This review is concerned with solvent components that play a role only at high concentrations. High concentration signifies a high molar ratio of the additive with respect to the primary solvent (e.g., water), such that the solvent properties diverge from those of the pure primary solvent. For example, enzyme reaction at low water activity has been conducted at near-100% organic solvent [1-5]. Numerous compounds, including salts, amino acids, polyhydric alcohols, and organic solvents, have been used in a co-solvent system [6-20], however it would not be feasible to cover all of these compounds and their applications in this review. The general observations of the effects of the co-solvent system on biological macromolecules will first be summarized, followed by a description of specific applications of co-solvent systems on biological macromolecules, including proteins, DNA, and viruses.

2. SOLVENT EFFECT OVERVIEW

The classical and widely used application of a co-solvent system is phase separation, or precipitation, of proteins and other macromolecules by salts and organic solvents. In 1888, Hofmeister reported an innovative observation that inorganic salts have a universal order in decreasing protein solubility [21]. While the mechanism behind this principle is still under extensive investigation (after over 100 years), a significant leap in mechanistic understanding was made by Traube in 1910 [22], when he reported that the "attraction pressure" of ions on water molecules significantly impacts the solubility of proteins, gases and other compounds. Hydration of ions leads to their repulsion from hydrated proteins, as both solutes tend to maximize their interactions with water molecules. The consequence of this repulsive effect is the thermodynamic destabilization of the entire system, resulting in phase separation or precipitation. There is an often-misquoted explanation that the ability of ions to adsorb water molecules depletes the hydration shell of the protein, exposing the protein surface for aggregation and precipitation. This is incorrect, as there are typically enough water molecules in the system to hydrate both proteins and salt ions, as long as the concentration is not too high (i.e., the two molecules are far apart). Furthermore, water desorption mechanism cannot explain the strong salting-out effects produced by the addition of organic solvents. The mechanism of salting-out is also through mutual repulsion between organic solvents and protein molecules, although the cause of repulsion is different from that operating in salt solutions [23].

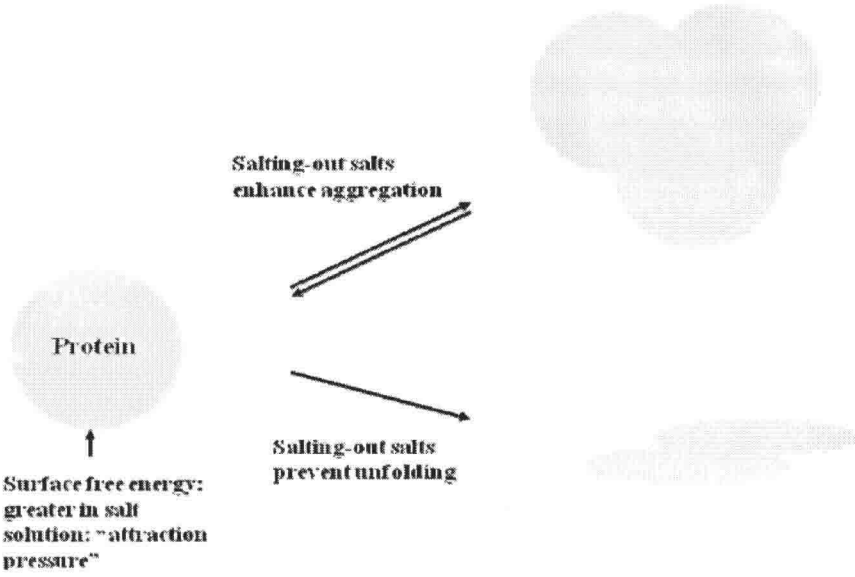


Figure 1. Illustration of cavity theory, demonstrating the various effects of salts on protein structure and stability.

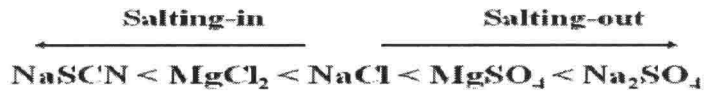


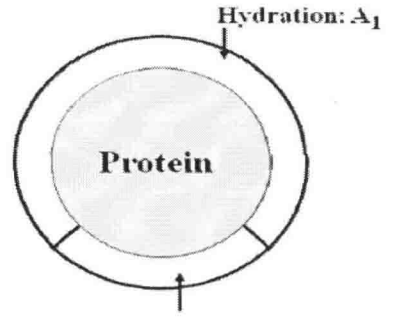
Figure 2. The order of salts according to their effects on macromolecules, either as a salting-in salt or salting-out salt.

The "attraction pressure" theory was further extended by Sinanoglu and Abdulnur [24], in the "cavity theory", to explain the effect of alcohol on DNA structure. They proposed that the surface tension is decreased by the addition of ethanol, which favors more solvent-exposed single stranded DNA, i.e., ethanol destabilized the DNA double helix. The "cavity theory" was further formalized by Melander and Horvath [25] to explain the effects of salts on protein solubility and its mechanism of action in hydrophobic interaction chromatography. According to the "cavity theory", the salts with high charge density have high "attraction pressure" on water molecules, and hence increase the surface tension of water effectively. This creates an unfavorable free energy around the surface of proteins and macromolecules, thus their association is one way by which the unfavorable free energy can be released (Figure 1). As shown in Figure1, reversible association of native proteins (here into a trimer) will lead to decreased surface area per protein, with consequent reduction in free energy associated with "attraction pressure", or in creating a cavity.

An alternative way to release the unfavorable free energy is to prevent protein expansion (unfolding). As shown in Figure1, unfolding exposes the interior of the protein to solvent molecules and increases the surface area (expressed as an elongated ellipsoid in the right column). This would result in increased free energy associated with the "attraction pressure". Thus, the salting-out salts stabilize the proteins and the salting-in salts destabilize them. The order of salts (see Figure 2), however, deviates at $MgCl_2$. Mg^{2+} effectively increases the surface tension of water due to its high charge density, but it is classified as a salting-in salt [26-30]. Such deviation is also observed with its effects on the other properties of proteins

and viruses [29-31], although the effects on virus stability are highly variable [32]. In all cases, the effects of $MgCl_2$ deviate from the trend observed in the "attraction pressure" principle. As described later, this deviation is due to the difference in the nature of protein-water interface and air-water interface. More specifically, protein surface is not a homogeneous non-polar surface, as is the case with air-water interface. The protein surface can attract certain co-solvents over water molecules, even those molecules that increase the surface tension of water. When salting-out salts are used to precipitate proteins, there is no danger of protein denaturation due to their stabilizing effects.

Water miscible organic solvents are strong protein precipitants [23, 29], and in general, they denature proteins at high concentrations [23, 29-38]. In fact, ethanol precipitation of plasma proteins is the most significant development in the realm of protein therapeutics, and has contributed greatly to the current progress in the production of recombinant biopharmaceuticals for a number of diseases and injuries that require blood component supplement [30, 39]. It is apparent that the effects of organic solvents on protein precipitation cannot be explained from the surface tension effects (cavity theory), as the addition of organic solvents decreases the surface tension [40-42]. Rather, the cavity theory explains the denaturing effects of organic solvents on the proteins, as described above for the destabilization of the DNA helical structure. How, then, does organic solvents decrease protein solubility on one hand and enhance unfolding on the other? This apparent paradox lies in the chemistry of the protein surface. The effects of salts can simply be related to the protein's surface area: salting-out salts favor the compact structure and salting-in salts favor the expanded structure. Organic solvents interact favorably with the expanded structure, with greater hydrophobic-exposed surface area, and unfavorably with the native structure, with polar residues solvent-exposed. There are two critical observations that can be made to explain the complex nature of organic solvents: 1) preferential interaction of organic solvents with proteins and 2) solubility of polar and non-polar compounds in organic solvents.



Bulk concentration: g_3

Domain concentration: $g`_3 = A_3/A_1$

Preferential interaction: $(\partial g_3/\partial g_2) = A_1(g`_3 - g_3)$

Figure 3. Illustration demonstrating the competing effects of water and co-solvent molecules for the surface of a protein.

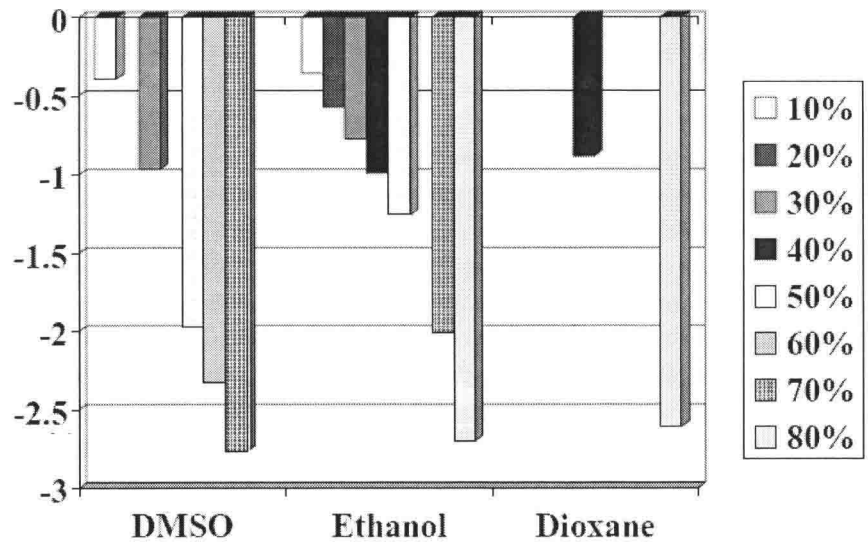


Figure 4. The solubility of glycine in various organic solvents, including DMSO (dimethyl sulfoxide), ethanol, and dioxane. The concentration of each solvents, ranging from 10 to 80%, is indicated in the figure.

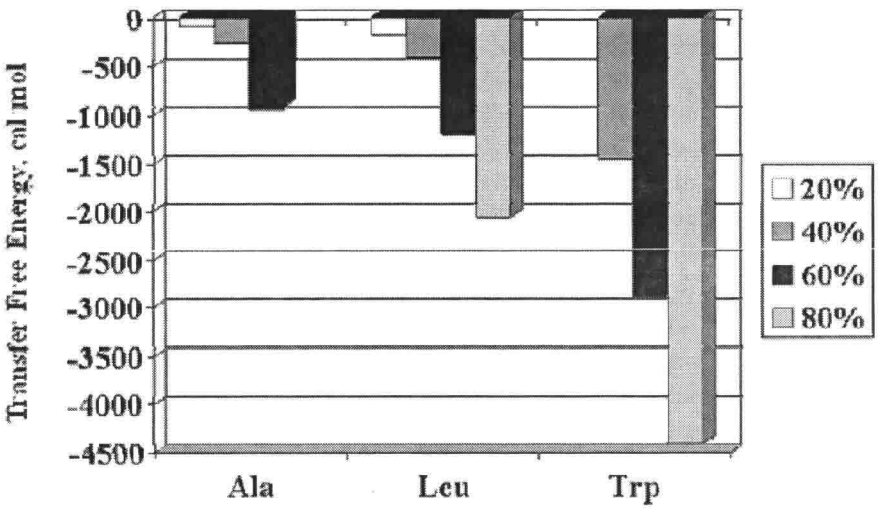


Figure 5. The transfer free energy of protein side chains, alanine (Ala), leucine (Leu), and tryptophan (Trp) in a DMSO co-solvent system, ranging in DMSO concentration from 20 to 80%.

A. Preferential interaction: Ligand binding to macromolecules can be readily determined by equilibrium dialysis. The co-solvents described here require high concentrations for their effects, suggesting that each individual interaction is weak, but can be large if sufficient number of bindings is present. In such case, the hydration of macromolecules contributes to the observed ligand binding as $(\partial g_3/\partial g_2) = A_1(g'_3 - g_3) = A_3 - g_3A_1$ where g_3 is the concentration of co-solvent in the bulk and g'_3 is the concentration of co-solvent at the protein surface, where both the properties of water and co-solvent are affected by the protein surface, as illustrated in Figure 3 [13, 18, 43, 44]. Their rotational and translational mobilities are

different from those in bulk solution. Formally, the observed ligand binding is the sum of co-solvent binding, A_3, and hydration, A_1. When g_3 (ligand concentration in binding experiment) is small, the second term (g_3A_1) is negligible. When ligand (co-solvent) concentration is high, the interaction of co-solvent with the protein is determined by both co-solvent binding and hydration, and hence is termed "preferential interaction" to indicate which solvent component, co-solvent or water, is preferentially bound to the protein. It has been observed that $(\partial g_3/\partial g_2)$ is negative in salting-out solutions, independent of the salt concentration, meaning that $g'_3 - g_3$ is negative and thus there is deficiency of salt concentration at the protein surface relative to the bulk concentration [45]. On the other hand, the salting-in salts have a tendency to bind to the proteins with $g'_3 - g_3 \geq 0$ [28, 30]. The negative interaction of salting-out salts correlates with the concept of "attraction pressure" in that the salts and the protein repel each other. On the other hand, salting-in salts show no correlation with the "attraction pressure". For salting-in salts, protein surface is not identical to the air-water interface and attracts the salts to its vicinity.

Organic solvents exhibit unique interactions with the protein surface. When the proteins are in the native structure, they are strongly excluded from the protein surface with $(\partial g_3/\partial g_2) = A_1(g'_3 - g_3) < 0$ [23, 38]. When the proteins are unfolded, they bind to the protein surface with $(\partial g_3/\partial g_2) = A_1(g'_3 - g_3) > 0$ [38]. For example, 2-chloro-2, 4-methylpentanediol (MPD), ethanol and dimethyl sulfoxide (DMSO) are all repelled from the native protein surface, although they bind to the proteins at high enough concentrations to unfold the proteins [23, 38]. This repulsion causes the protein to phase separate and precipitate, as has been observed previously [38]. This property of MPD or ethanol is used for protein crystallization or fractionation of plasma proteins. They are used, however, under optimal conditions, e.g., at intermediate concentration and low temperature, to avoid denaturation. A more strongly binding 2-chloroethanol demonstrates positive $(\partial g_3/\partial g_2)$, even at low concentrations, so that it cannot be used as a protein-precipitating agent [13, 18]. Instead, it is a good protein denaturing solvent, since it induces helical formation: it has been used to understand the helical structure of proteins.

B. Solubility: How does the effect of organic solvent depend on the chemistry of protein surface? The solubility data most clearly demonstrate the effects of organic solvents to cause phase separation, or decrease the solubility of polar compounds, and to increase the solubility of non-polar and aromatic groups. Strongly crystallizing MPD phase-separates upon the addition of ions, suggesting that MPD and electrical charges repel each other [23]. The solubility of polar glycine decreases in organic solvents, as shown in Figure 4 [38, 46]. In Figure 4, the ratio (in log scale) of glycine solubility in organic solvent relative to that in water is plotted against organic solvent concentration (10-80 %). The solubility decreases similarly in DMSO, ethanol and dioxane, indicating that the interactions of glycine with these organic solvents are highly, and similarly, unfavorable. On the other hand, the solubility of non-polar and aromatic groups increases with organic solvent concentration. Figure 5 shows the results of interaction of the side chain of alanine, leucine and tryptophan with DMSO (20-80%). The free energy is negative, indicating that the interaction is favorable. Thus, organic solvents are excluded (repelled) from the native protein with polar groups and charges that are solvent-exposed and bind to the non-polar and aromatic groups when they are solvent-exposed upon unfolding. This is why organic solvents can precipitate proteins in certain situations and cause denaturation in others.

The effects of organic solvents are schematically illustrated in Figure 6. When hydrated native protein is transferred into an aqueous solution containing low concentrations of organic solvents (upper panel), repulsion of protein molecules increases due to the decreased dielectric constant. In addition, lower dielectric constant may alter the ion distribution at the protein surface, and thus, protein hydration. However, the most significant effect of organic solvent is its repulsion from the charged protein surface, leading to an overall decreased solubility (that overwhelms the repulsion between protein molecules). At high concentration, organic solvents bind to the hydrophobic region of the unfolded protein. Such unfolding also causes decreased charge density of the protein, which in turn reduces repulsion between protein molecules (this effect should enhance protein association) and between protein charge and organic solvent (this effect should reduce protein association). Binding (solvation) of the unfolded structure by organic solvents should favor the dissociated state, suggesting that the effects of organic solvents on the solubility of unfolded protein may be unpredictable.

APPLICATIONS OF CO-SOLVENTS

1. Enhancing Protein Stability

In their natural environments, most biological macromolecules are stabilized by a number of factors. Unlike *in vitro* systems, they are not in dilute solution and instead are immersed in a highly concentrated macromolecular solution. Such crowded condition itself can stabilize proteins and enzymes [47-50]. They are often bound by other proteins and small molecules, which also stabilize these macromolecules. Alternatively, they may be made intrinsically unstable and hence designed for rapid turnover. In order to use these macromolecules for research purpose, drug development, and industrial application, they must be stabilized in the native, functional structure. Various salts, amino acids, amines, polyhydric alcohols, and sugars have been used to enhance the stability of proteins [6-20]. For example, Weisenberg and Timasheff made a pioneering observation when isolating brain microtubules [51]; the microtubules were readily purified from brain tissue homogenate by a simple temperature-controlled assembly and disassembly process [52, 53]. However, the microtubule proteins had to be recovered in the functional structure, which is maintained *in vivo* by microtubule-associated proteins (MAPs) [52, 53]. Prior to the reports by Weisenberg and Timasheff, the purified microtubules were always accompanied by incorporated MAPs, and it was assumed that the microtubule assembly required the presence of these proteins. This was proved to be incorrect when Weisenberg and Timasheff purified MAPs-free tubulins, which are subunit proteins of microtubules [51, 54, 55]. In other words, they purified tubulins in the absence of MAPs. What made this purification possible was the presence of a solvent additive, glycerol or sucrose, at a high concentration: ~30 % glycerol and ~1 M sucrose [54, 55]. Without these additives, tubulin lost the ability to polymerize into microtubules during purification and storage. More importantly, the purified tubulins were able to polymerize into microtubules in the presence of these solvent additives. Namely, these additives replaced the activity of MAPs, demonstrating that MAPs are not an essential factor for microtubule assembly.