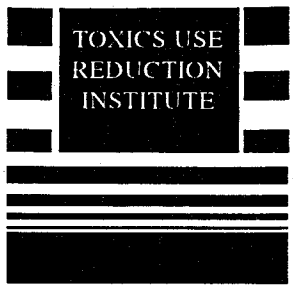

THE MASSACHUSETTS
TOXICS USE REDUCTION INSTITUTE

SUPERCRITICAL FLUIDS AS SUBSTITUTES FOR DRY CLEANING SOLVENTS:

EVALUATION OF ENZYME ACTIVITY FOR STAIN REMOVAL

Technical Report No. 28 **1995**

University of Massachusetts Lowell

Supercritical Fluids as Substitutes for Dry Cleaning Solvents: Evaluation of Enzyme Activity for Stain Removal

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The 1994 - 1995 Toxics Use Reduction Research Fellows Program

The Toxics Use Reduction Institute
University of Massachusetts Lowell

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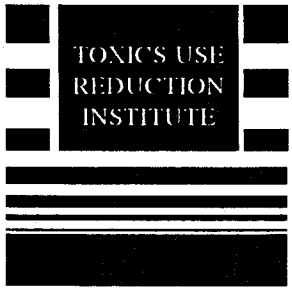


The Toxics Use Reduction Institute is a multi-disciplinary research, education, and policy center established by the Massachusetts Toxics Use Reduction Act of 1989. The Institute sponsors and conducts research, organizes education and training programs, and provides technical support to promote the reduction in the use of toxic chemicals or the generation of toxic chemical byproducts in industry and commerce. Further information can be obtained by writing the Toxics Use Reduction Institute, University of Massachusetts Lowell, One University Avenue, Lowell, Massachusetts 01854.

Toxics Use Reduction Institute
Research Fellows Program

In 1991, the Toxics Use Reduction Institute established the Research Fellows Program at the University of Massachusetts Lowell (UML). The Research Fellows Program funds toxics use reduction projects performed by a graduate student and his/her advisor. The goals of the Research Fellows Program are:

- to develop technologies, materials, processes, and methods for implementing toxics use reduction techniques
- to develop an understanding of toxics use reduction among UML graduate students and faculty
- to facilitate the integration of the concept of toxics use reduction into UML research projects
- to provide UML faculty with "incubator" funding for toxics use reduction related research, and
- to act as a liason between Massachusetts industries and UML faculty.

Notice

Supercritical Fluids as Substitutes for Cleaning Solvents :

Evaluation of Enzyme Activity in Supercritical Carbon Dioxide

for Stain Removal

SUMMARY

The toxicological and ecological hazards of perchloroethylene led the investigators to try supercritical carbon dioxide (SC CO_2) as a replacement for dry cleaning. Subtilisin Carlsberg (alkaline protease A) is used as a digesting enzyme with supercritical carbon dioxide. Supercritical carbon dioxide is a suitable nonaqueous solvent for the enzymatic hydrolysis of proteins. The Subtilisin Carlsberg exhibits activity in SC CO_2 at a wide pressure range from 1000 to 2500 PSI. Stability under supercritical conditions varies according to temperature and pressure. The enzyme is stable at 37°C for 1000-2500 PSI in SC CO_2. Addition of enzymes to supercritical carbon dioxide for dry cleaning would be highly desirable. Detailed systematic experiments have been carried out to optimize the enzyme's operational stability. The effect of pressure and temperature on the reaction have also been performed to assess the feasibility of using the enzymes for cleaning applications.

DESCRIPTION

1. Dry Cleaning

Perchloroethylene (PCE) is the most popular cleaning solvent used by dry cleaners. With more than 30,000 commercial shops in neighborhoods and malls across the country, dry cleaners are one of the largest groups of chemical users who are in direct contact with the public [1].

The dry cleaning process was developed to clean and control the shrinkage of fabrics. It is basically a method of washing clothes in a non-aqueous cleaning solution (though some water may be involved). The two dry cleaning solvents currently dominating the market in the United States are perchloroethylene and Stoddard Solvent. Other dry cleaning solvents with limited market penetration (CFC-113 and TCA) have been banned beginning in 1996, because of their potential to damage the ozone layer. Though other solvents have been examined as possible substitutes for dry cleaning, some of them have also been banned or are likely to be banned in the near future, due to their severe impact on the environment such as ozone depletion. One of the substitute solvents now in use in a few facilities is a petroleum solvent known as "140 Degree Solvent", which is a less flammable form of Stoddard Solvent. Other substitute solvents, including hydrofluorocarbons (HFCs) and fluorocarbons (FCs) continue to be explored, but possible commercial applications are farther in the future.

Because of the toxicological and ecological hazards of PCE, supercritical carbon dioxide (SC CO_2) is being investigated as a replacement for dry cleaning. Preliminary research [2] using polyester fabrics has indicated that supercritical carbon dioxide can effectively remove fatty substances from woven fabrics.

2. Supercritical Carbon Dioxide

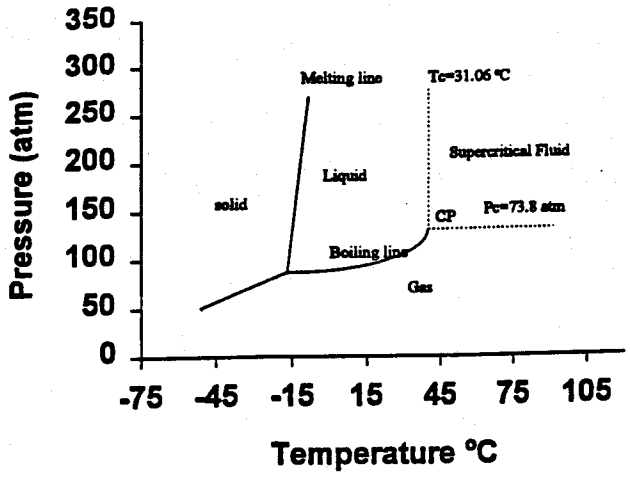


Figure 1. Phase diagram of carbon dioxide

Supercritical carbon dioxide has a critical temperature of 31.06 ^{o}C and a critical pressure of 73.8 atm as shown in Figure 1. Supercritical extraction has become a widely used method for the separation of various agents [3]. Like various available liquid extraction methods such as commercial degreasers, the solubility of various substances (impurities) in the supercritical phase is the key issue in determining efficiency. The food industry has been the largest user of this technique to accomplish tasks such as removing nicotine from tobacco, caffeine from coffee, etc. This technique can be further extended to separate a complex mixture into its various components-analogous to fractional distillation. A supercritical fluid can be further modified with the use of aqueous and non-aqueous solvents, e.g. alkanes, alcohols, ketones, water, to create a solvent phase with higher solubility characteristics than the supercritical fluid itself. Carbon dioxide is highly valued in such applications because it is inert and inexpensive. The application of supercritical carbon dioxide technology in areas requiring the use of expensive and/or toxic solvents is now becoming more widespread. Furthermore, the use of carbon dioxide facilitates 100% recycling and efficient toxic solvent use reduction.

Supercritical carbon dioxide as a replacement cleaning solvent offers a number of advantages over conventional liquid solvents. First, it is easy to tailor the solubility by changing temperature and/or pressure, depending on the needed application. In a certain range of temperature and/or pressure, the supercritical carbon dioxide has a significant cleaning effect, but neither destroys fabric nor dissolves dyes in the fabric. Another important characteristic of the supercritical carbon dioxide phase is the low viscosity and high diffusivity. It has the viscosity of a gas with extraction capabilities of a liquid. Thus, supercritical carbon dioxide possesses the ability to clean in small cavities, such as beneath and between the yarns and fibers of a cloth. Furthermore, the carbon dioxide is nonpolar and is expected to dissolve grease easily. In the conventional dry cleaning process, the solvent entrapped in clothes can leave an irritating odor in the cleaned garment. Since carbon dioxide leaves no residue in clothes after depressurizing to atmospheric pressure, this problem is resolved.

As discussed earlier, SC CO_2 has several potential advantages over conventional solvents for dry cleaning processes. However, it also has some potential limitations such as the high pressures (500-4000 PSI) that may be needed to achieve cleaning and create the need for expensive high-pressure equipment for safe operating conditions.

3. Effect of Pressure on Enzymes

Chemical or biochemical reactions in supercritical fluids (SCF) are conducted at high pressures. Transition-state analysis is introduced here to explain the rate enhancement observed at high pressure in SCF. (Note that there may exist additional, indirect effects of pressure.)

According to transition-state theory, the rate constant k, may be calculated using Boltzmann's constant, k_B, Planck's constant, h, and gas constant, R:

$$k = r(\frac{k_B T}{h}) \exp(-\frac{\Delta G^{\mu}}{RT}) \quad (1)$$

$$= r(\frac{k_B T}{h}) \exp(-\frac{\Delta G^{\mu}}{RT}) \exp(-\frac{\Delta G^{\mu}}{RT}) \quad (2)$$

where ΔG^{μ}, ΔH^{μ} and ΔS^{μ} are transition-state activation free energy, enthalpy and entropy respectively, and r is the transition coefficient. The relationship between ΔG^{μ} and ΔV^{μ}, and the rate constant based on molar concentration depends on pressure as follows:

$$(\frac{\delta \ln k}{\delta P}) = -\frac{\Delta V^{\mu}}{RT} + \beta_o \Sigma v_i \quad (3)$$

where v_i is the stoichiometric coefficient (negative for reactants and positive for products), β_o is the compressibility coefficient of solvent, and ΔV^{μ} is the activation volume (defined as the difference between the volume of activated complex and the volume of initial reactants). The unusual reaction behavior of ethylene polymerization in supercritical ethylene, for example, has been ascribed to a very large negative partial molar volume of the activated complex near the critical point of the solvent.

The effect of pressure on an enzymatic reaction may also be determined by considering the activation volume ΔV^{μ} consisting of two parts: volume change of the catalytic step, ΔV_c^{μ}, and of the binding step, ΔV_b^{μ}:

$$\Delta V^{\mu} = \Delta V_c^{\mu} + [K_m / (K_m + C)] \Delta V_b^{\mu} \quad (4)$$

where C and K_m are the concentration of the substrate and the Michaelis-Menten constant, respectively. The temperature and pressure dependence of the activation volume ΔV^{μ} can be expressed (if the free energy change of the reaction is considered) as :

$$\Delta V^{\mu} = \Delta V_o^{\mu} + \Delta\beta(P-P_o) + \Delta\alpha(T-T_o) \quad (5)$$

The volume changes ΔV_c^{μ} and ΔV_b^{μ} were measured at pressures of up to 4000 psi for several proteases. If both values are negative, this indicates that the reaction rate is enhanced at high pressures.

Protein denaturation which leads to enzyme inactivation may also be considered as a "reaction" and is influenced not only by temperature, but also by pressure. However, the

pressure to which protein is subjected in SCF such as SC CO_2 is less than the pressure reported to cause protein denaturation in water.

4. Detergent Enzymes in Supercritical Carbon Dioxide (SC CO_2)

Supercritical carbon dioxide (like most other dry cleaning solvents) is a very good natural solvent for oils and greases, but is less effective in dissolving water soluble contaminants (e.g. proteins). Effective cleaning in supercritical carbon dioxide will require the removal of a wide variety of contaminants such as food, grass and blood, as well as difficult-to-clean materials such as paint and ink. Enzymes are commonly employed to remove such stains from clothing in aqueous based detergents. These active materials "digest" the components of a stain, allowing for its removal. A recent work [4] has shown that enzymes can also be viable in organic solvents. We speculate that enzymes can also be employed in a solvent such as SC CO_2 to accomplish similar tasks. Actually, in conventional dry cleaning processes, about 2% of moisturized proteolytic enzyme is added which is enough to achieve desired cleaning.

In the past decade, various influencing factors such as activity [5], stability [6], and specificity [7] of enzymes in anhydrous organic solvents [8] have been investigated. The achieved results initiated the development of interesting applications such as the syntheses of pharmaceuticals, chiral intermediates, special polymers, and in our proposal, the potential for stain removal during dry cleaning. Subtilisin Carlsberg was chosen for these studies for several reasons: (i) the enzyme is cofactor-independent, hence, cofactor-enzyme interactions need not be considered ; (ii) Subtilisin Carlsberg (alkaline protease A) is a serine protease and is widely used as an additive in detergents as a digesting enzyme; (iii) It is active in a wide variety of organic solvents and is catalytically active even with very little bound-water [ca. 9% (wt./wt.)] [9]. These properties make it ideally suited for use in conjunction with SC CO_2 to remove stains of proteinaceous origin.

Chemical reactions in supercritical carbon dioxide have been investigated for many years. Enzymatic catalysis in SC CO_2, first demonstrated in 1985, is now receiving widespread attention. In addition to reduced mass-transport limitations and simplified separations, supercritical carbon dioxide offers several other potential advantages for enzymatic reactions. Since SC CO_2 does not solubilize enzymes, separation and recovery of the enzyme after the reaction is easier. Nonpolar substrates such as greases are more soluble in supercritical carbon dioxide than in aqueous solutions, and the reduced water activity attainable in SC CO_2 may permit the reversal of many hydrolytic reactions. For example, cholesterol is 50 times more soluble in supercritical carbon dioxide (at 123 bar and 35°C) than in water. Furthermore, the critical temperature of carbon dioxide is 31°C, low enough for the processing of many heat-labile fabrics.

Our key goal in the evaluation of supercritical carbon dioxide as a replacement for perchloroethylene in dry cleaning is to develop an understanding of enzyme activity in supercritical fluids, especially in supercritical carbon dioxide. Work also focuses on

developing enzymes with the needed chemical and physical stability to function in supercritical carbon dioxide. Evaluation of the stain removal capabilities will be performed, when enzymes stable in supercritical carbon dioxide have been developed. This novel work will significantly aid in the adoption of environmentally friendly carbon dioxide for use in the industrial dry cleaning process.

RESEARCH METHODOLOGY

1. Materials

Subtilisin Carlsberg (from *Bacillus licheniformis*) was obtained from Sigma (Sigma Chemical Co., St. Louis, MO 63178). The enzyme was activated prior to use in supercritical carbon dioxide by lyophilization from aqueous 20 mM phosphate buffer (pH 7.8). In this manner, the functional groups of enzyme are retained in their catalytically active form in nonaqueous media. All other chemicals and solvents used in this work were of the highest grade commercially available. All solvents were twice distilled and stored over molecular sieves at least 24 hrs prior to use.

2. High Pressure Reactor

Figure 2 shows a schematic diagram of the high-pressure reactor which has been designed and constructed for biocatalytic reactions and extractions in supercritical fluids at the University of Massachusetts Lowell. In order to ensure efficient stirring of the reactants and to connect an on-line UV detector to monitor enzyme activity, it is important to have a "view cell". This high pressure optical cell has an internal volume of 45 ml with a pressure rating of 7500 psi, and a temperature rating of 100°C. This cell is constructed with sapphire windows for added strength and resistance to chemical degradation. A batch system is used in order to keep a constant amount of water during the reaction. Temperature is maintained with a K-type thermocouple and an Omega controller. Pressure is determined with a pressure transducer. The pressure and temperature of the entire system are monitored and controlled by a computer.

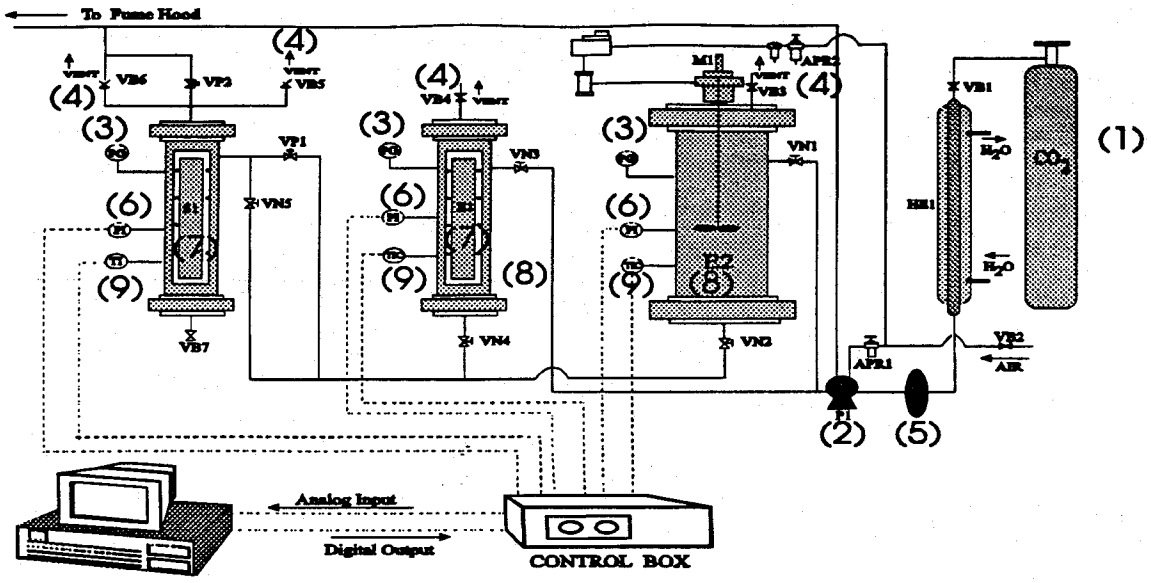
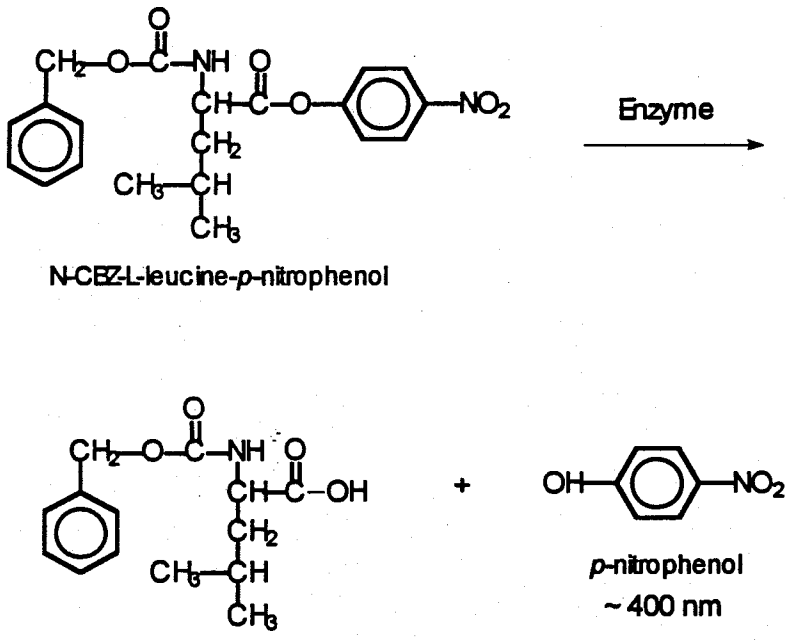


Figure 2. Reactor configuration for high-pressure biocatalytic reactions (1) gas cylinder; (2) high pressure pump; (3) pressure gauge; (4) rupture disc; (5) filter; (6) pressure transducer; (7) high pressure optical cell with sapphire window; (8) stainless steel reactor; (9) temperature readout

3. Measurement of Enzyme Stability and Activity

(1) Standardization of the enzyme activity in aqueous solutions

Preparation of standard curve of enzyme activity in aqueous solutions was measured in stirred glass reactors at room temperature. To solubilize N-carbobenzoxy (CBZ)-L-leucine-*p*-nitrophenyl ester as substrate, 5% v/v of dimethylsulfoxide was added to a 50-mM phosphate buffer (pH 8.0). N-CBZ-L-leucine-*p*-nitrophenyl ester was added to make a 1-mM solution. Reactions were initiated by adding substrate dissolved in the above solution and 1% wt/v of powder enzyme. The enzymatic activity of subtilisin can be determined by the colorimetric method. The principle of the colorimetric method depends on the determination of *p*-nitrophenol released rate in solution at 400 nm upon hydrolysis of N-CBZ-L-leucine-*p*-nitrophenyl ester at pH 8.0 by subtilisin, with correction for any nonenzymatic (spontaneous) hydrolysis of the substrate. Because of the broad range of hydrolytic activity of this enzyme and the difficulties in predicting its proteolytic activity, this hydrolytic rate was chosen as a general indicator of subtilisin enzyme behavior.



(2) Incubation in supercritical carbon dioxide :

Known amounts of freeze dried enzyme in glass test tubes were incubated in supercritical carbon dioxide using the apparatus shown in Figure 2. The incubation was performed at varied pressures and temperature. The depressurization step was performed as slowly as possible (5-10 min.). Incubated enzyme was then dissolved in a phosphate buffer solution. Their enzyme activities were determined spectrophotometrically at 400 nm at room temperature.

RESULTS AND DISCUSSIONS

1. Enzyme activity in supercritical carbon dioxide

Since carbon dioxide is one of the most commonly used supercritical fluids, we have initiated our investigation of the enzyme-catalyzed reaction on hydrolysis of N-CBZ-L-leucine-*p*-nitrophenyl ester in supercritical carbon dioxide. Given the results in Figure 3, we were not surprised to find that the reaction in carbon dioxide proceeds well.

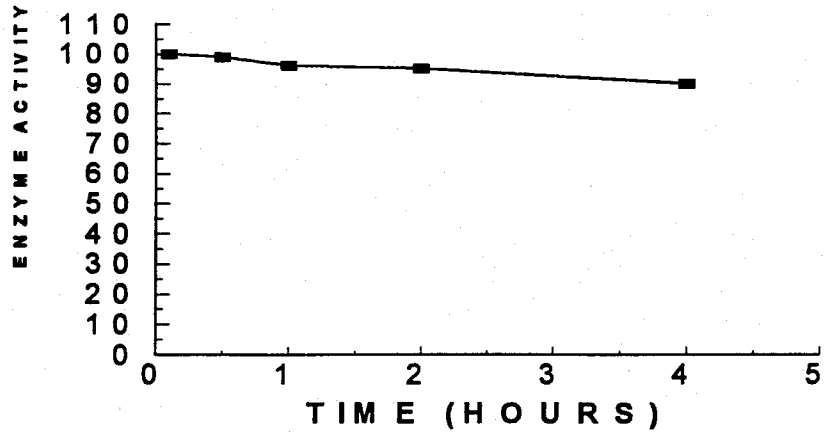


Figure 3. Batch reaction kinetics of N-CBZ-L-leucine-p-nitrophenyl ester with Subtilisin Carlsberg (from *Bacillus licheniformis*) in supercritical carbon dioxide at 37^{0}C, 1500 PSI

The utility of a catalyst in an industrial reaction is closely related to its stability and recyclability. We have investigated the ability of protease to catalyze hydrolysis of esters at high temperatures for a longer period of time. The enzyme is stable at 37°C and 1000-2500 PSI in supercritical fluids. The enzyme retains essentially all its activity as shown in Fig. 4.

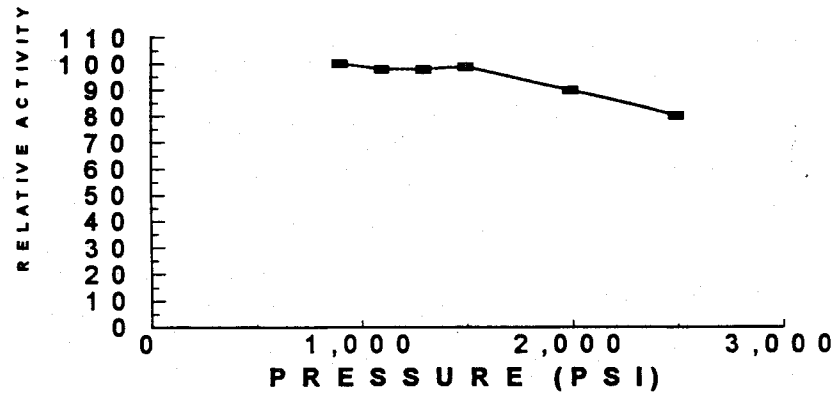


Figure 4. Effect of pressure on the enzyme activity in supercritical carbon dioxide at 37^{0}C for a constant treatment time of 30 min.

Given our interest in the activity of enzymes in SC CO_2, which is essentially the only supercritical fluid utilized in biocatalytic reactions to date, we were interested in the effect of carbon dioxide on the hydrolysis reactions. Figure 4 demonstrates that high pressure carbon dioxide inhibits the protease-catalyzed hydrolysis of esters to some degree. This finding is not surprising, given the expected interaction between a protein powder and carbon dioxide. First, carbon dioxide will form carbamates with many primary and secondary amines, and thus lysine residues on the surface of the protein can undergo covalent modification in the presence of carbon dioxide. Second, it is well known that suspended enzyme particles are associated with bound water molecules. The presence of carbon dioxide would be expected to lower the effective pH on the surface of the enzyme and thus could inactivate the protein. This possibly harmful complexation and pH effect of carbon dioxide on enzyme activity in supercritical fluids media have not been discussed previously.

$$CO_2 + RNH_2 \rightarrow \underset{\text{carbamates}}{RNH\text{-}COOH}$$

$$CO_2 + H_2O \rightarrow \underset{\text{acid}}{H_2CO_3}$$

CONCLUSIONS

We have demonstrated for the first time that SC CO_2 is a suitable nonaqueous solvent for the enzymatic hydrolysis of esters using Subtilisin Carlsberg enzyme. These studies demonstrate that the enzyme is stable under conditions that are thought to be suitable for carbon dioxide dry cleaning applications.

For a useful enzymatic cleaning agent to be realized, a number of additional studies would be desired. For example, the amount of water in the system significantly affects enzyme activity, and detailed studies examining water's effect need to be performed. Additionally, the useful lifetime of the enzyme under SC conditions needs to be determined to ascertain the economic feasibility of enzyme additives in SC cleaning processes. Lastly, future work should be directed towards developing other enzymes that can be used in conjunction with Subtilisin for the removal of a greater number of staining substances.

Acknowledgment

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