Activities of Several Enzymes in Ethanol + Water at Elevated Pressure of Carbon Dioxide

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The activities of cellulase, α-amylase, and acid protease in ethanol + water at elevated CO_2 pressure were investigated. The experimental results indicated that the operating conditions of under 30% (mass percent) ethanol concentration and lower than 35 °C were quite suitable for cellulase−ethanol−water systems using high-pressure CO_2, but α-amylase treated with high-pressure CO_2 was liable to denature. The results also showed that it was suitable to precipitate acid protease in high-pressure CO_2 (upward 6.86 MPa) within a wide range of ethanol concentration (upward 50%) and temperature (under 60 °C).

1. Introduction

It is an extraordinarily prospective and practical novel technique to extract rare valuable products from aqueous solutions using high-pressure or supercritical CO_2 instead of an organic solvent or organic solvent mixture that does great harm to the environment. Supercritical CO_2 extraction is probably one of the earlier and best-known applications of CO_2 in separation processing. Recently, supercritical CO_2 antisolvent has been developed and is becoming an attracting research field.[1]

In fact, precipitation as one of the typical methods of biological materials isolation remains an indispensable unit operation. Up to 80% of published protein purification protocols include at least one precipitation step, ranging from primarily isolating (removing cellular debris from recombinant cell cultures) to obtaining a protein product solution with reduced volume, even the refinement of the product prior to drying and formulation.[2] Precipitation processes have the advantages of rapidly isolating protein products and reducing their exposure to unfavorable conditions and proteolytic enzymes. In recent years, studies on high pressure or supercritical CO_2 are noticeable. Tomasula et al.[3−5] precipitated casein from milk in batch and continuous processes using high-pressure CO_2 at 38 °C and 5.5 MPa and investigated the solubility of CO_2 in milk in detail. The researchers in Holland isolated soybean protein from its raw mixture, where the volatile electrolyte CO_2 was used as a precipitant. The process produced no wastewater with acid or alkaline at all and could be regarded as "green separation technology".

Biological materials are generally sensitive to heat, chemical conditions, and even shearing forces (stirring) and tend to degrade and denature.[6] Proteins usually lose their activities seriously during conventional organic solvent precipitation. As for precipitation with supercritical CO_2, whether proteins will recover their full or a majority of biological activities is the precondition to succeed this process. The stability of enzyme in supercritical fluids has been described in the scientific literature since the mid 1980s. Various studies[1,7] have reported the influence of several parameters such as temperature, pressure, moisture in the SC−CO_2, and pressurization and depressurization steps on the stability and the catalytic activity of enzymes in SC−CO_2. Otherwise, the papers about enzymatic stability in supercritical fluids to get a desired product, focused on using supercritical CO_2 as an antisolvent (supercritical antisolvent, SAS) to induce rapid protein precipitation from an organic solvent (e.g., DMSO),[2,8] and few similar reports were found about precipitation of protein from aqueous phase.

Our preliminary experiments showed that protein aqueous solution was hard to deposit until higher pressure was reached when compressed CO_2 was introduced directly, while the pressure of CO_2 for protein deposition would be relatively lower if some organic solvents such as ethanol or acetone were added properly into protein aqueous solution in advance. The stability of protein or enzyme in high-pressure or supercritical CO_2 is the key factor in the precipitation of protein using high-pressure or supercritical CO_2. If proteins or enzymes are inactive and unstable, the method will not come true. Therefore, the law of enzyme activities based upon the precipitation of protein using high-pressure CO_2 will be investigated in the present work. The effects of CO_2 pressure and ethanol on activities of cellulase, α-amylase, and acid protease will be discussed.

2. Materials and Methods

2.1. Materials. Cellulase, which was provided by the Institute of Bioengineering in Zhejiang University, was produced by solid-state fermentation. Raw cellulase was prepared by spray drying after soaking and ammonium sulfate precipitation, and its specific activity is about 2000 IU/g. α-Amylase was purchased from Wuxi Enzyme Preparation Factory, and its specific activity is about 6000 IU/g. Acid protease was provided by the Institute of Microorganism, Academy of Agricultural Sciences in Zhejiang province, and its specific activity ranges from 300 to 350 IU/g. Several enzymes mentioned above were stable not only at ambient conditions but also in experimental ethanol−water solutions under atmospheric pressure. The gaseous CO_2 of a purity of 98% was provided by the Gas Supply Station in Zhejiang University. All other analytical chemicals were used, with no further purification.

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10.1021/je049938o CCC: $27.50
Published on Web 07/22/2004

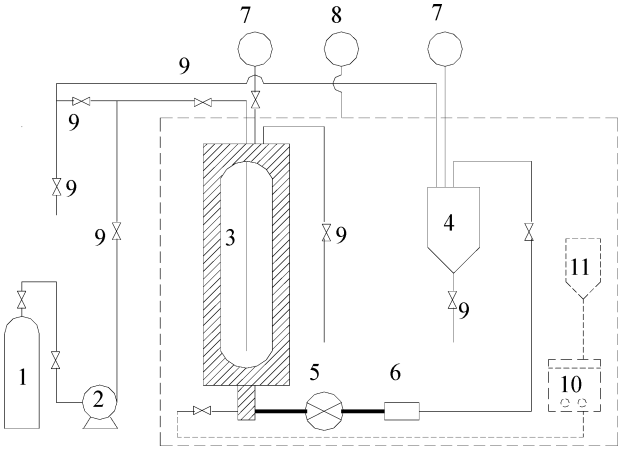

Figure 1. Schematic diagram of the experimental apparatus. 1, carbon dioxide cylinder; 2, high-pressure pump; 3, precipitation vessel; 4, separation vessel; 5, ball valve; 6, filter; 7, pressure gauge; 8 temperature controller; 9. cutoff valve; 10, peristaltic pump; 11, feed vessel.

2.2. Assay for Enzyme Biological Activity. Cellulase activity was measured for saccharification according to the method recommended by refs 9 and 10. The enzyme can hydrolyze carboxymethyl cellulose (CMC) into glucose whose quantity is determined, and then endoglucanase (EC3.2.1.4) activity was given. Bernfeld assays (see also ref 11 in detail) were used for α-amylase activity measurement. The quantity of reductive substance (maltose referred) was determined in this method. Acid protease activity was measured according to the Extraction Chem. Co. method (improved Amano method) recommended by ref 11. The quantities of amino acid and peptide (tyrosine referred), which yielded from protease hydrolyzing casein and cannot be precipitated by TCA (trichloroacetic acid), are determined. The activities of cellulase, α-amylase, and acid protease were expressed as relative activities compared with their activities in original feeds (100%).

2.3. Apparatus and Operation. The apparatus, shown in Figure 1, consists of a high-pressure precipitation vessel with a window, CO_2 antisolvent supply system, feed unit, separation unit, and air-bath system. The CO_2 inlet was set at the top, and the feed inlet and outlet was at the bottom. The CO_2 antisolvent supply system was made up of a CO_2 cylinder and two cascade mini high-pressure metering pumps (WZJ-2, Zhi-Jiang Science Instrument Factory, Zhejiang Province). Feeding unit, which could be removed after feeding, was composed of a feed tank and a peristaltic pump (HL-1, Shanghai Hu-Xi Instrument Factory). The separation unit contains a ball valve, a filter, and a gas−liquid separator. The temperature of the air chamber is regulated by a temperature controller through electric heaters and circulating fans. The precipitation vessel, CO_2 antisolvent inlet lines, the feed unit, and the separation unit are housed within an insulated air chamber.

The experiments were operated in a batch mode. First, CO_2 was introduced via the CO_2 inlet of the precipitation vessel and discharged via the outlet several times to purge the remaining air. Second, a known quantity of enzyme was dissolved in a certain mass-percent ethanol−water mixtures, and then the stock solution was delivered to the precipitation vessel already preheated. Typically ~30−40 mL of solution was added. Following thermal equilibration, compressed CO_2 was continuously and slowly added to the bottom of the precipitation vessel and attained a certain pressure point predetermined. After that, the pressure was kept constant and the change of solution into suspension was examined through the window of the vessel. The temperature of the precipitation vessel remained constant during the process. After keeping pressure for 0.5 h, the thoroughly mixed liquid suspension was sampled from feed inlet. After dilution, the activities of samples were measured. Then the operation mentioned above was repeated till the tiptop of the operating pressure.

Every experiment was repeated in triplicate, and the average values were adopted.

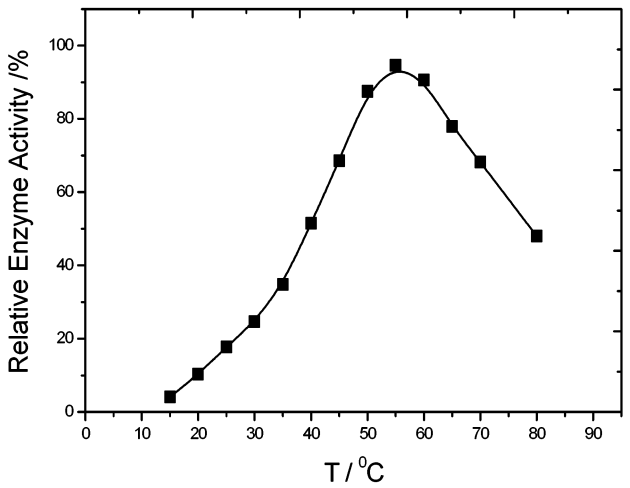

Figure 2. Effect of temperature on the activity of cellulase atmospheric pressure (1% crude enzyme, no buffer).

3. Results and Discussion

Separating a target protein from the culture medium requires specific conditions. The protein will lose its biologic activities, and its biologic half-life will also decrease rapidly, if the specific condition cannot be satisfied.[12] Therefore, it is necessary and a key point to perform preliminary experiments for understanding essential characteristics and ascertaining fractionation conditions before the investigation of protein properties.

3.1. Experimental Conditions Selected and Influence of Operating Conditions on Activity of Cellulase. 3.1.1. Experimental Conditions. First the influence of temperature (from 15 to 80 °C) on reaction and stability of cellulase was investigated, because temperature always plays great impacts on the stability of enzyme. Cellulase activity was measured at different temperatures (as shown in Figure 2). The results illustrated that the optimum temperature of cellulase catalysis is about 55 °C, and the enzyme is stable below 60 °C. When the temperature was increased above 60 °C, crude enzyme solution was incubated for different time intervals, and the activities were measured (as shown in Figure 3). The recovered activities of enzyme incubated for 1 h and 2 h at 65 °C and at 70 °C are 68.5%, 59.0% and 62.3%, 45.6%, respectively. Thus it can be concluded that cellulase is highly thermostable. Finally, temperature from 20 to 40 °C and ethanol content from 10 to 40% in ethanol−water solutions were selected as experimental conditions. The concentration of cellulase was fixed at 1%.

3.1.2. Influence of Operating Conditions on Activity of Cellulase. 3.1.2.1 Temperature. The effects of different ethanol concentrations and CO_2 pressures on enzyme activities at 20 °C, 30 °C, 35 °C, and 40 °C were illustrated in Figures 4−7 and Table 1. Enzyme activities were expressed as relative activities that were based upon 100% of cellulase activities in original feeds. From Figures 4−7 and Table 1, there is no quantitative correlation between

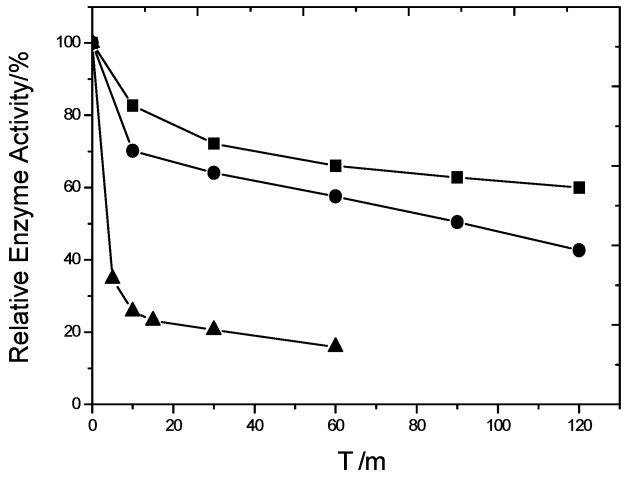

Figure 3. The stability of cellulase at different temperature atmospheric pressure (1% crude enzyme, no buffer): ■, 65 °C; ●, 70°C; ▲, 80 °C.

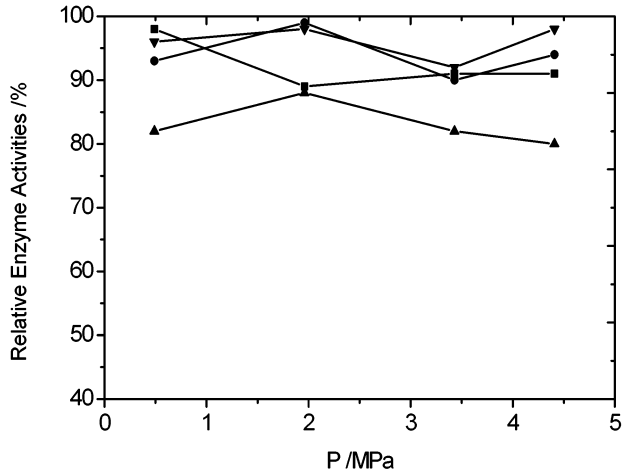

Figure 4. Activities of cellulase in ethanol−water solution at high-pressure CO_2 under 20 °C: ■, 40% ethanol; ●, 30% ethanol; ▲, 20% ethanol; ▼, 10% ethanol.

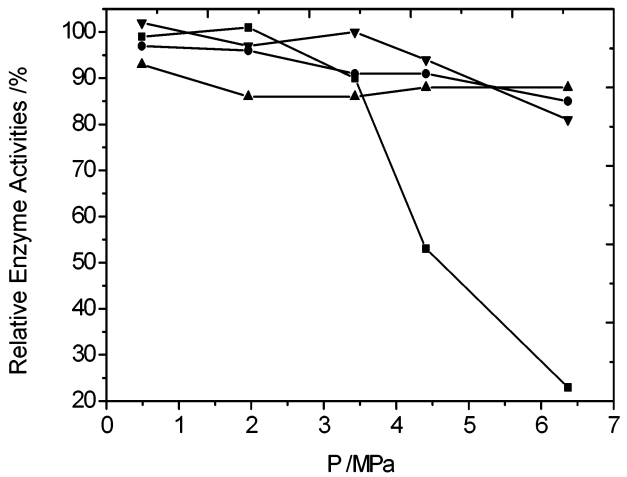

Figure 5. Activities of cellulase in ethanol−water solution at high-pressure CO_2 under 30 °C: ■, 40% ethanol; ●, 30% ethanol; ▲, 20% ethanol; ▼, 10% ethanol.

operating conditions and recovered activities at 20 °C, but the activities fall with the increase of CO_2 pressure and ethanol concentration at higher temperatures. At conditions of 4.41 MPa, the recovered activities in solutions with 30% ethanol are 80% and 11% at 35 °C and 40 °C,

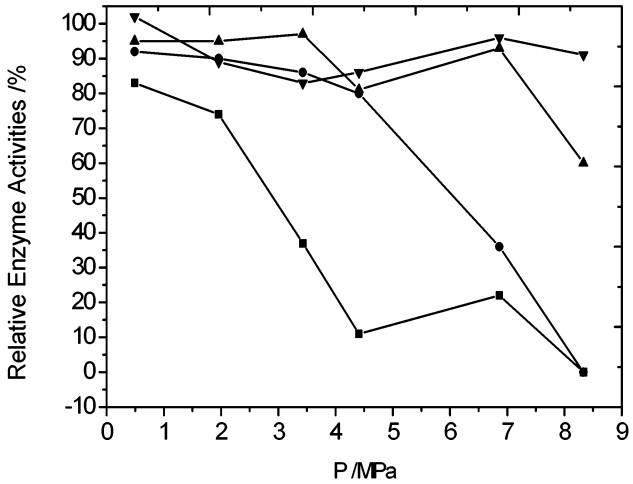

Figure 6. Activities of cellulase in ethanol−water solution at high-pressure CO_2 under 35 °C: ■, 40% ethanol; ●, 30% ethanol; ▲, 20% ethanol; ▼, 10% ethanol.

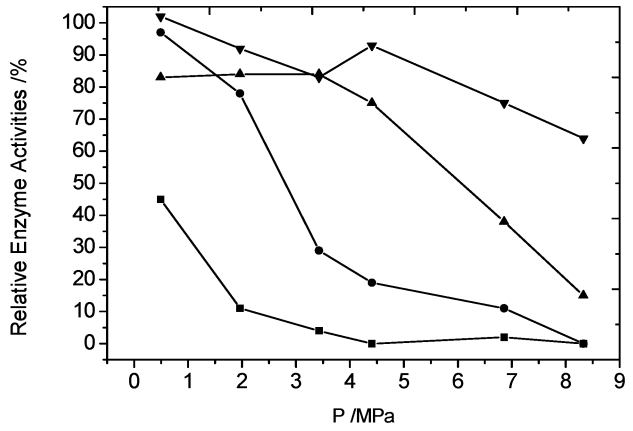

Figure 7. Activities of cellulase in ethanol−water solution at high-pressure CO_2 under 40 °C: ■, 40% ethanol; ●, 30% ethanol; ▲, 20% ethanol; ▼, 10% ethanol.

Table 1. Relative Activities of Cellulase in Ethanol−Water Solutions at Different Pressures of CO_2

ethanol concentration/%	*T*/°C	pressure of CO_2/ MPa 0.49	1.96	3.43	4.41	6.37	8.33
10	20	96	98	92	98		
	30	102	97	100	94	81	
	35	102	89	83	86	96	91
	40	102	92	83	93	75	64
20	20	82	88	82	80		
	30	93	86	86	88	88	
	35	95	95	97	81	93	60
	40	83	84	84	75	38	15
30	20	93	99	90	94		
	30	97	96	91	91	85	
	35	92	90	86	80	36	0
	40	97	78	29	19	11	0
40	20	98	89	91	91		
	30	99	101	90	53	23	
	35	83	74	37	11	22	0
	40	45	11	4	0	2	0

respectively, and are only 19% and 0% in solutions with 40% ethanol. That means the enzyme deactivated completely under this condition.

Thus it can be seen that the activities remained high with the rising of pressure and ethanol concentration at lower temperature but lost greatly at higher temperature. Therefore, temperature is a very important factor in this process and lower temperature is helpful to the process.

3.1.2.2. Ethanol Concentration. The effects of different temperatures and CO_2 pressures on enzyme activities at ethanol concentrations of 10%, 20%, 30%, and 40% were deduced in Figures 4−7 and Table 1. For solutions with different ethanol content, the recovered activities in 10% ethanol solution are nearly the same despite of the increase of pressure (<4.41 MPa) at 20 °C to 40 °C. In solutions with 20% ethanol, the recovered activities do not vary with the elevation of pressure at 20 °C, 30 °C, and 35 °C but fall sharply with pressure upward 3.43 MPa at 40 °C. In solution with 30% ethanol, the recovered activities keep constant with pressure rising at 20 °C and 30 °C but decrease obviously at 40 °C and with pressure upward 3.43 MPa at 35 °C. In solution with 40% ethanol, the recovered activities kept nearly the same with pressure rising only at 20 °C, but fall greatly at 30 °C, 35 °C, and 40 °C.

All in all, the influence of ethanol concentration on activity of cellulase is much more complicated. The recovered activities do not vary obviously with the change of temperature and pressure at lower ethanol concentration but fall greatly with ethanol concentration surpassing 40%. The enzyme is activated by the cooperative effects among lower ethanol concentration (10%), higher temperature (35 °C), and pressure (4.41 MPa), also among higher ethanol concentration (40 wt %), lower temperature (20 °C), and higher pressure (3.43, 4.41 MPa), which should be utilized and paid great attention to.

3.1.2.3. CO_2 Pressure. As was illustrated in Figures 4−7 and Table 1, the recovered activities in solutions with 30% and 40% ethanol fall obviously with the increase of temperature at 3.43 MPa and 4.41 MPa and are well above 80% and insusceptible in other ethanol concentration solution. Figure 5 shows that, operated at 6.37 MPa and 30 °C, the recovered activities are almost above 80% in solutions with ethanol content less than 30% but are only 23% and decreased sharply in the case of 40% ethanol content. Figure 6 shows that operated at 6.86 MPa and 35 °C, the recovered activities are 90% in solutions with ethanol content less than 20% and only 36% corresponding enzyme activities recovered in solution with 30% ethanol. While operated at 8.33 MPa, the recovered activities are 60%, 0%, and 0% in solutions with 20%, 30%, and 40% ethanol, respectively. The results of Figure 7 illustrate that operated at 6.68 and 8.33 MPa and 40 °C the recovered activities are always below 80% and decrease obviously with the increase of ethanol concentration. Therefore, avoiding operation at high pressure in higher temperature and ethanol concentration is suggested to recover activities.

The experimental results indicate that CO_2 pressure affects the recovered activities only at higher temperature (40 °C) and that ethanol concentration (40%) and is not the key factor on the loss of enzyme activities. The probability is provided to precipitate protein with high-pressure CO_2 at mild conditions of lower ethanol concentration. The results also imply that the effects of temperature, pressure, and ethanol concentration are highly interrelated.

In brief, the activities of cellulase in ethanol−water solution (ethanol concentration from 10% to 40%) were measured at temperatures of 20 to 40 °C and at pressures of 0.49 to 8.33 MPa and we can conclude:

(1) Temperature and ethanol concentration are important factors on the recovered activities in this process and exert great impacts at higher temperature. Particularly, the recovered activities are above 80%, except for at 40% ethanol concentration above 30 °C, 30% ethanol concentration at 40 °C, and pressure more than 0.49 MPa. The process should be away from higher temperature, higher pressure, and higher ethanol concentration.

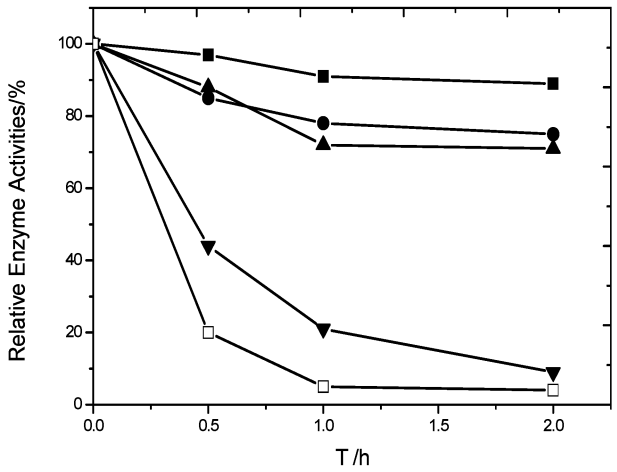

Figure 8. Activities of α-amylase in aqueous solution under 40−100 °C atmospheric pressure (1% crude enzyme, no buffer): ■, 40 °C; ●, 50 °C; ▲, 60 °C; ▼, 70 °C; □, 100 °C.

(2) CO_2 pressure is the subordinate factor on the loss of enzyme activities. Operating at less than 30% ethanol concentration and lower than 35 °C is not markedly influencing on the recovered activities, but elevation of CO_2 pressure is helpful to the depositing process.

3.2. Experimental Conditions Selected and Influence of Operating Conditions on Activity of α-Amylase. 3.2.1. Experimental Conditions. The results of the preliminary experiments about the thermostability of α-amylase were displayed in Figure 8. Figure 8 explains that α-amylase is stable at 40−60 °C and keeps pretty high activities even incubated for 2 h. α-Amylase will lose its activity in 0.5 h if it was in temperatures higher than 65 °C. Temperatures of 20−50 °C and ethanol content of 10% to 40% were selected as experimental conditions. The concentration of α-amylase was fixed at 1%.

3.2.2. Influence of Operating Conditions on Activity of α-Amylase. The experiments of α-amylase similar to cellulase were carried at temperatures of 20−50 °C and pressures of 0.49−8.33 MPa in solutions with 10% to 40% ethanol.

3.2.2.1. Temperature. The effects of different temperatures and CO_2 pressures on α-amylase activities in solutions with 10%, 20%, 30%, or 40% ethanol were shown in Figures 9−12 and Table 2. As illustrated in the figures, the recovered activities decrease greatly with the increase of temperature. Operating at lower temperature such as 20 °C, the recovered activities in solutions with 10% to 40% ethanol are more than 50%, but the activities fall greatly at higher temperature such as 30 °C, 35 °C, 40 °C, and 50 °C. In particular, operating at higher temperature and CO_2 pressure even in solutions of lower ethanol concentration, such as at 40 °C and 50 °C, with 10% and 20% ethanol, the recovered activities at more than 1.96 MPa are less than 26% and lost tremendously.

3.2.2.2. Ethanol Concentration. Figure 9 shows that the recovered activity in solution with 10% ethanol falls greatly with the increase of pressure except a certain pressure point at 20 °C. Figure 10 illustrates that the recovered activity in solution with 20% ethanol falls inferior to that in the case of 10% ethanol except at 30 °C and 35 °C. Figures 11 and 12 explain that the recovered activities in solutions with 30% and 40% ethanol have the same downtrend at 20 °C and 30 °C and fall complexly and

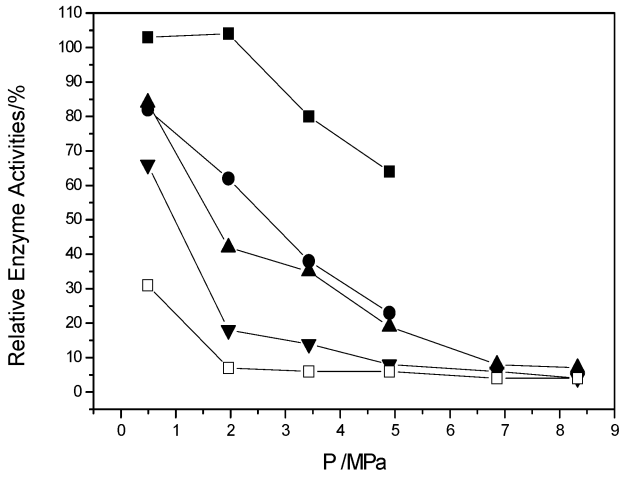

Figure 9. Activities of α-amylase in 10% ethanol−water mixtures at high-pressure CO_2: ■, 20 °C; ●, 30 °C; ▲, 35 °C; ▼, 40 °C; □, 50 °C.

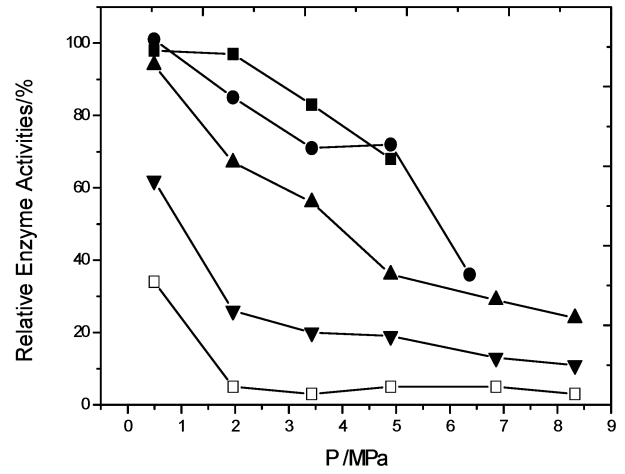

Figure 10. Activities of α-amylase in 20% ethanol−water mixtures at high-pressure CO_2: ■, 20 °C; ●, 30 °C; ▲, 35 °C; ▼, 40 °C; □, 50 °C.

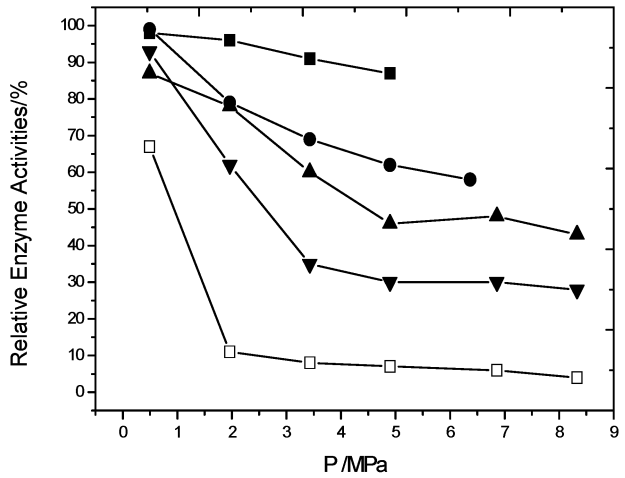

Figure 11. Activities of α-amylase in 30% ethanol−water mixtures at high-pressure CO_2: ■, 20 °C; ●, 30 °C; ▲, 35 °C; ▼, 40 °C; □, 50 °C.

differently at 35 °C, 40 °C, and 50 °C. It is quite unusual that the recovered α-amylase activities in solutions of lower ethanol concentration fall faster than that in solutions of higher ethanol concentration with the increase of CO_2 pressure.

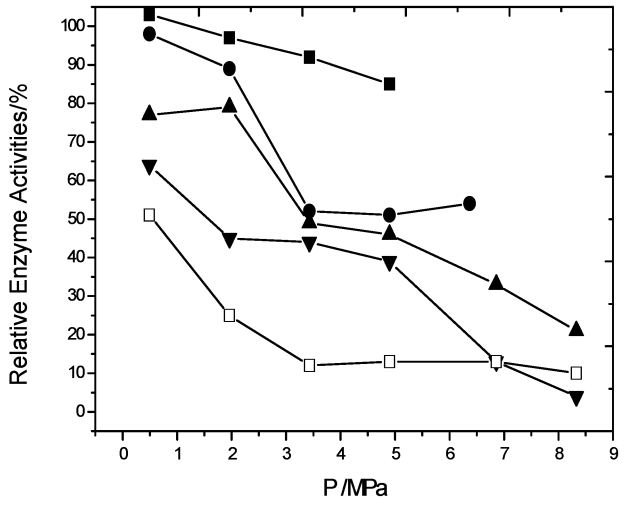

Figure 12. Activities of α-amylase in 40% ethanol−water mixtures at high-pressure CO_2: ■, 20 °C; ●, 30 °C; ▲, 35 °C; ▼, 40 °C; □, 50 °C.

Table 2. Relative Activities of α-Amylase in Ethanol−Water Solutions at Different Pressures of CO_2

ethanol concentration/%	*T*/°C	pressure of CO_2/ MPa 0.49	1.96	3.43	4.41	6.37	8.33
10	20	103	104	80	64		
	30	82	62	38	23	15	
	35	84	42	35	19	8	7
	40	66	18	14	8	6	4
	50	31	7	6	6	4	4
20	20	98	97	83	68		
	30	101	85	71	72	36	
	35	74	67	56	36	29	24
	40	62	26	20	19	13	11
	50	34	5	3	5	5	3
30	20	98	96	91	87		
	30	99	79	69	62	58	
	35	87	78	60	46	48	43
	40	93	62	35	30	30	28
	50	67	11	8	7	6	4
40	20	103	97	92	85		
	30	98	89	52	51	54	
	35	77	79	49	46	33	21
	40	64	45	44	39	13	4
	50	51	25	12	13	13	10

3.2.2.3. CO_2 Pressure. Figures 9−12 and Table 2 show that the recovered activities decrease greatly with the increase of CO_2 pressure and lose completely in particular at 6.86 MPa and 8.33 MPa or in solutions with 10% ethanol. The recovered activities at 20 °C and 30 °C except for in solutions with 10% ethanol fall slowly with the increase of pressure and are above 50% even at 4.9 MPa and 6.37 MPa. Moreover, the recovered activities at other temperatures fall rapidly. For instance, the recovered activities at 50 °C go down to 25% when the pressure only runs up to 1.96 MPa.

Therefore, we deem that CO_2 pressure is the key factor on the loss of enzyme activities especially when temperature is rising constantly and that it is difficult to deal with α-amylase by high-pressure CO_2 except at lower temperature such as 20 °C.

3.3. Influence of Operating Conditions on Activity of Acid Protease. The relative enzyme activity is almost immovable when acid protease aqueous solution was incubated in 80 °C and 100 °C water baths for 2 h. The relative activities of acid protease in ethanol−water solution of 40% and 50% ethanol content incubated for 1−3 h in a 100 °C water bath were shown in Figure 13. The

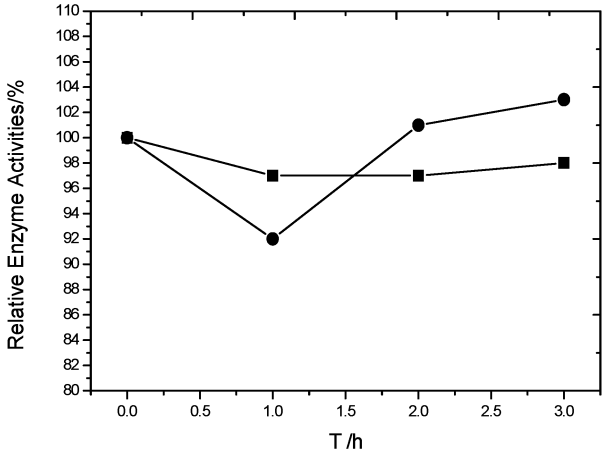

Figure 13. Relative activities of acid protease in ethanol−water solution under 100 °C atmospheric pressure: ■, 50% ethanol; ●, 40% ethanol.

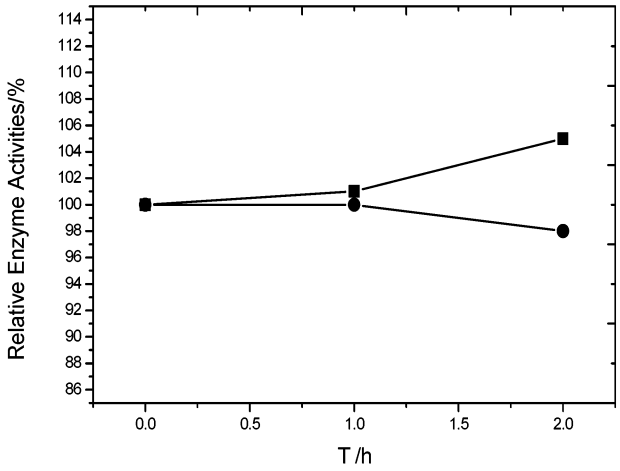

Figure 14. Effect of incubative time on the activity of acid proteasein ethanol−water solution at 60 °C, 6.86 Mpa: ■, 50% ethanol; ●, 40% ethanol.

results indicate that the recovered activities are between 92% and 101% and that acid protease is highly thermostable, obviously. The variations of relative acid protease activity in ethanol−water solution of 40% and 50% ethanol incubated for 1−2 h in 6.86 MPa and 60 °C were depicted in Figure 14. Figure 14 illuminates that the relative enzyme activity is in the range of 98% to 105% and that acid protease has a high stability of CO_2 pressure.

Temperature of 60 °C, ethanol concentration of 10% and 40%, and acid protease of 1% were selected as experimental conditions according to the preliminary tests of acid protease. Figure 15 shows that the recovered activities at the experimental conditions mentioned above are 99% to 106% and that the deposition emerges in 40% ethanol concentration solution with the increase of CO_2 pressure.

Therefore, we think that acid protease is not sensitive to temperature, CO_2 pressure, and ethanol concentration, and it is suitable to treat solutions of acid protease with high-pressure CO_2 (upward 6.86 MPa) within a wide range of ethanol concentration (upward 50%) and temperature (below 60 °C).

3.4. Precipitation Phenomena. The extent of depositions would be observed through the precipitation vessel window after compressed CO_2 was introduced into different ethanol−water stock solutions of cellulase, α-amylase, and acid protease at different temperatures. The speed and the amount of deposition appeared are: cellulase > α-amylase > acid protease. The starting pressure of CO_2 for deposition from ethanol−water mixtures falls with the increase of temperature and ethanol concentration, taking solutions of cellulase as an example (See Table 3). The amount of precipitation increases with the rising of temperature, ethanol concentration, and CO_2 pressure.

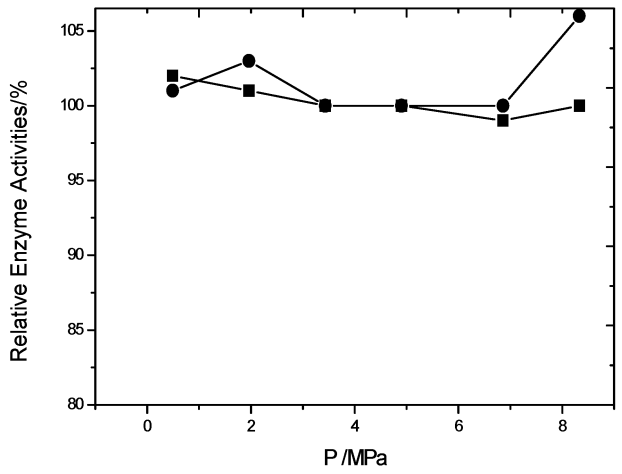

Figure 15. Activities of acid protease in 10% and 40% ethanol−water solution at high-pressure CO_2 under 60 °C: ■, 10% ethanol; ●, 40% ethanol.

Table 3. Starting Pressure of CO_2 for Deposition in Cellulase−Ethanol−Water Solutions

	T/°C	starting pressure for deposition/MPa
10% ethanol−water solution	20	no deposition
	30	4.41
	35	3.43
	40	1.96
20% ethanol−water solution	20	4.41
	30	3.43
	35	1.96
	40	0.49
30% ethanol−water solution	20	1.96
	30	0.49
	35	0.49
	40	0.49
40% ethanol−water solution	20	0.49
	30	0.49
	35	0.49
	40	0.49

3.5. Phase Equilibria and the Experimental Deviation. It is necessary to study the phase equilibria of this system at different conditions of pressure, temperature, and solution compositions because the phase behavior will influence the state of enzyme solutions and the values of relative enzyme activities. For this research, we assumed that the enzyme solution was similar to the ternary systems containing CO_2, C_2H_5OH, and H_2O and regarded that biomolecules hardly affected the phase equilibria of $CO_2-C_2H_5OH-H_2O$. The reasons are that the substances except for CO_2, C_2H_5OH, and H_2O are involatile, hydrophilic, and fewer in quantity.

In the past decade, the phase equilibria for the $CO_2-C_2H_5OH-H_2O$ ternary system have been studied by many researchers,[1,13−16] because ethanol owes its important potential applications in biochemical processes and can be used as a model compound for fundamental research in supercritical fluid extraction. According to the results and experimental data (shown in Table 4) of Yao et al.,[15] the solubility of ethanol and water in $SC-CO_2$ is lower than 0.015 in mole fraction (P < 7.85 MPa, x_3^0/x_2^0 < 2.55) and x_3/x_2 (the ratio of the mole fraction of water to ethanol in

Table 4. Results of Phase Equilibrium Data for CO_2(1) + C_2H_5OH(2) + H_2O(3) Systems

		SCF phase composition					
T/K	*P*/MPa	y_1	y_2	y_3	x_3^0/x_2^0 in feed[a]	x_3/x_2 cal[b]	ethanol % in feed
308.15	7.85	0.9965	0.0019	0.0016		23.61	
	6.86	0.9980	0.0008	0.0012	23	23.62	10
	4.71	0.9986	0.0003	0.0011		23.64	
308.15	7.85	0.9930	0.0051	0.0019		10.46	
	6.86	0.9966	0.0020	0.0014	10.22	10.47	20
	4.90	0.9971	0.0012	0.0017		10.49	
323.15	7.85	0.9938	0.0038	0.0024		10.50	
	6.86	0.9952	0.0027	0.0021	10.22	10.51	20
	4.90	0.9953	0.0019	0.0028		10.52	
313.15	9.81	0.9838	0.0125	0.0037	9.0	9.45	
	4.9	0.9948	0.0040	0.0012		9.47	22
308.15	7.85	0.9907	0.0068	0.0025		6.34	
	6.86	0.9950	0.0034	0.0016	5.96	6.34	
	4.90	0.9964	0.0017	0.0019		6.35	30
308.15	7.85	0.9874	0.0098	0.0028		2.66	
	6.86	0.9940	0.0042	0.0018	2.55	2.66	50
	4.90	0.9954	0.0022	0.0024		2.66	

[a] x_3^0/x_2^0 is the ratio of the mole fraction of water to ethanol in the liquid feed. [b] x_3/x_2 is the ratio of the mole fraction of water to ethanol in the liquid after equilibrium.

the liquid after equilibrium) will be equal to x_3^0/x_2^0 (the ratio of the mole fraction of water to ethanol in the liquid feed) approximately after equilibrium. So we conclude that the equilibria have little effect on liquid phase and enzymes.

At the same time, the maximal mean deviation of relative enzyme activities in this work is within ±5.2%. Therefore, the change of enzyme solutions influenced by phase equilibria will not have a great impact on measuring the values of relative enzyme activities and we ignored influence of phase equilibria in this work.

4. Conclusion

The following conclusions can be obtained from the activities having been measured of cellulase, α-amylase, and acid protease at elevated CO_2 pressure:

(1) For cellulase, CO_2 pressure is not the key factor on the loss of enzyme activities, but temperature and ethanol concentration play a great impact on the recovered activities. The cellulase−ethanol−water system can be operated by compressed CO_2 at less than 30% ethanol concentration and lower than 35 °C.

(2) For α-amylase, any of CO_2 pressure, temperature, and ethanol concentration can induce the loss of activities. It is difficult to deal with α-amylase by high-pressure CO_2 except at lower temperature (20 °C) and pressure (<1.96 MPa).

(3) For acid protease, the recovered activities are almost constant at high temperature (under 60 °C), high CO_2 pressure (upward 6.86 MPa), and high ethanol concentration (upward 50%). Thus, it is suitable to treat acid protease solutions of high ethanol concentration, at high-pressure CO_2, and high temperature.

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Received for review February 7, 2004. Accepted June 10, 2004. This work was financially supported by the National Natural Science Foundation of China (Nos. 29736180 and 20076042).

JE049938O