Proteinase-catalyzed Hydrolysis of Casein at Atmospheric Pressure and in Supercritical Media

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Dedicated to Prof. Dr. Đurđa Vasić-Rački on occasion of her 60th birthday

In the presented work, reaction parameters for hydrolysis of casein, catalyzed by *Carica papaya* latex at atmospheric and high pressure, were optimized. Casein is a remarkably efficient nutrient, supplying not only essential amino acids, but also some carbohydrates, calcium, phosphorus and therefore is very important for the food industry.

Different reaction parameters such as temperature, stirring rate, casein and enzyme concentration were studied to found the optimal conditions for the reaction. Reactions were performed at atmospheric pressure; an influence of temperature/pressure on the casein hydrolysis in supercritical carbon dioxide (SC CO_2) was also investigated to improve the reaction rates. Higher conversions were achieved when the reactions were performed in SC CO_2, even though casein was not soluble in this medium.

Key words:
Enzymatic reaction, supercritical carbon dioxide, high-pressure, proteinase, *Carica papaya* latex

Introduction

The most important protein in bovine milk is *casein* which precipitates under acid conditions. It is insoluble at pH = 4.6 (isoelectric point), and has a molecular weight of 34 kDa.[1] Casein is not a single substance, but a family of phosphorus-containing proteins that bind calcium and other minerals present.

Most of casein in milk is in the form of *casein micelles*, aggregates of several thousands of casein molecules with a diameter of 10–300 nm. The detailed structure of those micelles is not known with certainty but it is generally assumed that it comprises a roughly spherical aggregate of the α- and β-caseins surrounded by a coating of κ-casein.[2]

Traditionally, acidic protein hydrolysis processes have been used for the production of complete protein hydrolysates; the processes have high efficiency and low operations cost, but destroy the acid-sensitive amino acids and introduce salt into the products. That is the reason why the applications of this method are limited in the field of the food industry.[3]

Protein hydrolysis using enzyme as a biocatalyst could avoid most of these problems.

Proteases from a number of plants including fig (*Ficus carica*), cardoon (*Cynara cardunculus* L.), paw paw (*Carica papaya*), pineapple (*Ananas sativa*) and castor oil seeds (*Ricinus communis*) have been found to coagulate milk.[4]

Papain (EC 3.4.22.2) is a thiol proteinase obtained from *Carica papaya* latex, which is of great importance in brewing, food, and pharmaceutical industries. It degrades a wide range of protein substrates, splitting them into peptides and amino acids.[5]

The polypeptide chain of papain contains 212 amino acid residues and its molecular weight is 23 kDa. This enzyme includes a sequence of asparagine, histidine and alanine at the active site. Essential to the active site is a cysteine at position 25 in the sequence of the enzyme, and six other cysteines help to maintain the conformation of papain through disulphide bridges.[5]

The use of supercritical fluids (SCFs) as solvents in chemical synthesis has environmental, health, safety and chemical benefits.[6] Environmental benefits of most SCFs in industrial processes are in the replacement of environmentally far more damaging conventional organic solvents. An environmental impact is also found in low energy consumption during operation.[7] SC CO_2 has been widely used as an enzymatic reaction medium in the last few decades. The interest to combine the biocatalyst and SC CO_2 as a reaction medium has been growing rapidly in recent years. Such systems become more and more interesting also for industrial and pilot applications.[8,9]

Dense gases and particularly supercritical or near-critical fluids have some advantageous properties over organic solvents, such as tuneable solvation ability and the possibility of eliminating solvent residues. Furthermore, the use of SC CO_2 decreases mass transfer limitations, because of the high diffusivity of reactants in supercritical medium, low surface tension and because of the relatively low viscosity of the mixture. For the application of enzymes as biocatalysts in compressed gases they must have a good stability and activity in those media.[10,11]

One of the expected benefits from using enzymes in SCFs is that mass transfer resistance between the reaction mixture and the active sites in the solid enzyme can be greatly reduced.[6,12]

The aim of our research work was to optimize the reaction parameters of casein hydrolysis at atmospheric and high pressure. As a biocatalyst, proteinase from *Carica papaya* latex was used.

Effects of temperature, stirring, casein and enzyme amount were investigated at atmospheric pressure. Experiments were carried out not only at atmospheric pressure but also under supercritical conditions in a high-pressure batch stirred tank reactor (HP BSTR) in SC CO_2, where reactions at different temperature/pressure combinations were carried out to improve the reaction rates.

Experimental procedures

Enzyme preparation

Proteinase: Proteinase preparation Promod™ 144P from *Carica papaya* latex was kindly donated from Biocatalysts, England.

Chemicals

Casein (No. 102241), potassium di-hydrogen orthophosphate-anhydrous (KH_2PO_4), di-sodium hydrogen orthophosphate-di-hydrate ($Na_2HPO_4 \cdot 2H_2O$) and trichloroacetic acid (TCA) were from Merck, Germany.

Carbon dioxide (99.95 % by volume pure) was supplied by Messer MG Ruše, Slovenia.

Phosphate buffer (pH = 7)

0.157 g of KH_2PO_4 and 1.575 g of $Na_2HPO_4 \cdot 2\ H_2O$ was dissolved in about 50 mL of deionized water; the solution was diluted with water to the total volume of 100 mL in a volumetric flask.

Casein solutions of different concentrations

Since casein is just slightly soluble in water at 20 °C, adding of phosphate buffer (pH = 7) was followed by vigorous stirring and heating up to 60 °C until complete dissolution. Then the casein solution was cooled down to the ambient temperature. After cooling the solution remained homogeneous.

Analytical method

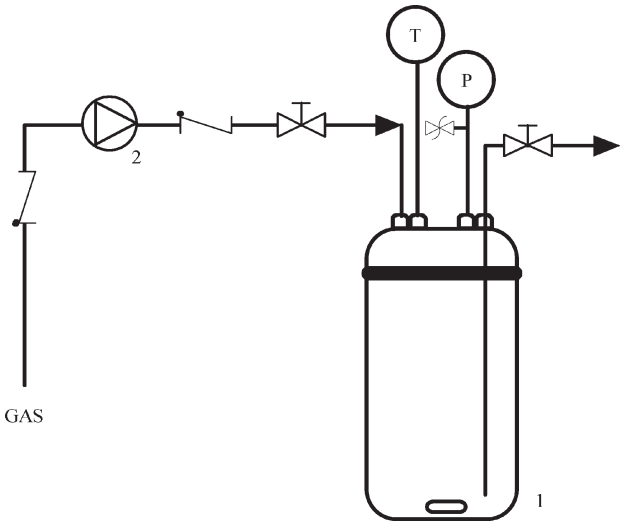
The samples were taken from the reaction mixture at present times and the extent of proteolysis was determined by a spectrophotometric method.

3 mL of TCA was added to 2 mL of the sample and mixed thoroughly. After leaving for 30 minutes at ambient temperature it was centrifuged at 4000 rpm for 20 minutes. The absorbance of the supernatant was read against deionized water using Varian Cary 100 type spectrophotometer at 280 nm.

For blank sample, 2 mL of casein solution was mixed with 3 mL of TCA and being left at ambient temperature followed by centrifugation (4000 rpm, 20 min). It can be regarded as a 'zero sample' taken from the reactor.

Proteolysis in batch stirred tank reactor at atmospheric pressure (BSTR) and in SC CO_2 (HP BSTR)

The design of the batch-operated system is shown in Fig. 1. The volume of the reactor, which was designed for operation at 500 bar and 200 °C, was 88 mL.



F i g . 1 – *Design of batch-stirred-tank reactor (BSTR) for enzyme-catalyzed reaction performed at atmospheric pressure or under high pressure. 1 – reactor; 2 – high pressure pump; P – pressure indicator; T – temperature indicator and regulator.*

Casein solution was added into the reactor and thermostated at the desired temperature. A magnetic stirrer and heater were used to ensure a homogeneous reaction mixture and hold the temperature constant. The blank sample was taken from the reactor mixture before the papain solution was added.

When the reaction was performed at high pressure, CO_2 was pumped from the cylinder into the reactor by a high-pressure pump immediately after addition of the biocatalyst. At preset times samples were taken from the reaction mixture and analyzed according to the method described above. The sample withdrawal had minimal influence on the pressure in the reactor.

Each data point represents the average of at least two measurements or the average of three measurements, when the problems with operation at high pressure appeared.

Results and discussion

The enzyme-catalyzed hydrolysis the casein is based on splitting of casein molecules into free amino acids. The production of free amino acids could be improved with optimization of reaction parameters. The influence of reaction parameters on casein hydrolysis was investigated in a BSTR at atmospheric and high pressures.

Optimization of reaction parameters for proteinase-catalyzed casein hydrolysis at atmospheric pressure

Influence of enzyme concentration

A primary objective of any treatment system should be to minimize the cost. Usually the enzymes represent one of the main cost components and therefore the concentration in the reaction mixture should be minimized. Concentration of the biocatalyst also has a great influence on the conversion. Therefore the influence of concentration of proteinase from *Carica papaya* latex on equilibrium conversion was studied.

Hydrolysis of casein with proteinase from *Carica papaya* latex in BSTR was done at 40 °C and enzyme concentration was varied. The concentration of casein was 10 g/L of reaction mixture in all experiments. The influence of proteinase concentration on production of protein hydrolysates after 2 minutes and after 4 hours of reaction is presented in Table 1.

T a b l e 1 – *Influence of enzyme concentration on conversion for enzyme catalyzed casein hydrolysis. Table presents conversions at defined enzyme concentrations in dependence on the reaction time.*

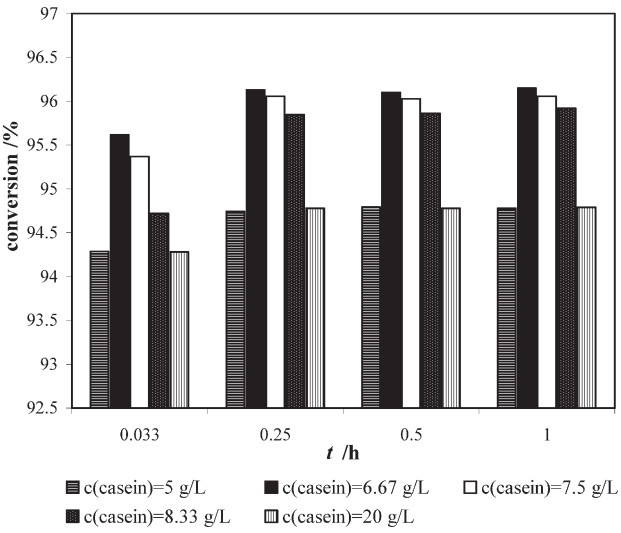
t/h	Enzyme concentration/(g/L)			
	2.5 g/L	10 g/L	30 g/L	40 g/L
0.033	92.417	95.506	96.051	96.079
4	93.074	96.097	96.091	96.100

The maximal conversion of 96 % was detected, when the concentration of enzyme was 10 g/L. A lower initial reaction rate was observed at lower biocatalyst concentration (2.5 g/L), according to the conversion data after 2 minutes of reaction. The conversion after 4 hours of the reaction at the same biocatalyst concentration was about 93 %. With further increase in enzyme concentration over the optimal concentration a slight increase in initial reaction rates was perceived, but no significant differences in conversion after 4 hours of reaction were observed. Therefore the concentration 10 g/L of proteinase from *Carica papaya* latex was used as optimal enzyme concentration for all further experiments, because of the minimum cost principle.

Influence of substrate concentration

The dependence of conversion on the substrate concentration was also studied. The reactions were performed at 40 °C, proteinase concentration of 10 g/L and mixer stirring rate of 600 rpm. Concentration of substrate – casein was varied from 5 g/L to 20 g/L.

In Fig. 2 it can be seen that the maximal initial rate was found at casein concentration of 6.67 g/L, where the conversion after one hour of reaction was 96.11 %.

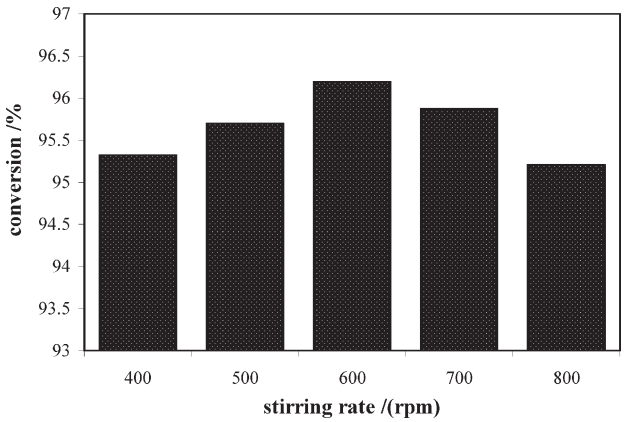


F i g . 2 – *Conversion of casein at different substrate concentrations, in the presence of proteinase from Carica papaya latex. Reaction parameters were: temperature; 40 °C, proteinase concentration; 10 g/L and stirring rate; 600 rpm.*

The equilibrium of reaction was achieved already after 1 hour of the reaction. At higher and lower casein concentrations, lower conversions were obtained. The reason for lower conversions at higher casein concentrations (e.g. 20 g/L) could be in saturation of reaction mixture with substrate. Therefore the contact between active site of enzyme and substrate could be hindered.

Determination of the optimal stirring rate

The influence of stirring rate on conversion of casein at atmospheric pressure, 40 °C, enzyme concentration of 10 g/L and substrate concentration of 6.67 g/L was investigated. The results are presented in Fig. 3. The highest conversion after 4 hours of the reaction was observed at stirring rate of 600 rpm. Between 400 rpm and 600 rpm an increase in conversion was perceived.



F i g . 3 – *Effect of stirring rate on the conversion of casein in enzyme-catalyzed hydrolysis at 40 °C, at optimal concentrations of enzyme and substrate and at atmospheric pressure after 4 hours of the reaction*

Both casein and papain are large molecules, dispersed in water. Therefore the reaction mixture can hardly be considered homogeneous at molecular level.

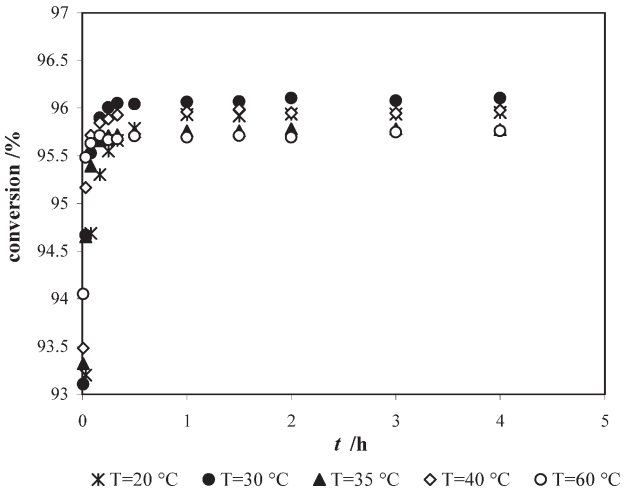
At lower stirring rates, the mass transfer was limited. The contact between substrate from the bulk liquid and enzyme surface, where the active site of enzyme is placed, was prevented or limited.

At 700 rpm and 800 rpm the conversion decreased, because of high shear forces, which disturbed the molecular shape of the enzyme. Consecutively denaturation of enzyme occurred. Casein and papain molecules are of comparable size. Therefore one should expect that shear forces could have caused the denaturation of casein, too.

Temperature influence and determination of thermodynamic parameters

Two effects are joined during the increase in temperature; reaction rate increases with higher temperature on one hand and enzyme activation/deactivation occurs on the other hand.[13]

Reactions were performed in the range between 20 °C and 60 °C. The determined optimal temperature was 30 °C (Fig. 4).

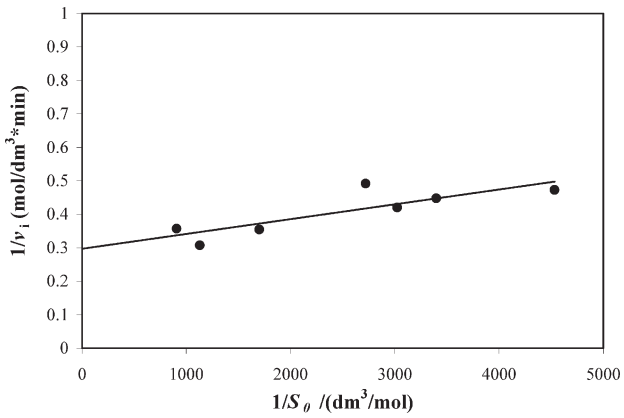


F i g . 4 – *Effect of temperature on the conversion vs. time profile for enzyme-catalyzed casein hydrolysis, at optimal concentrations of enzyme and substrate and at atmospheric pressure*

When the reaction was performed at 60 °C, the initial reaction rate was the highest (Fig. 4) but obviously with the time, the enzyme was slightly deactivated and therefore the conversion after 4 hours of reaction was very low, quite comparable to that of 35 °C.

From Arrhenius plot (not shown), which represents the dependence of logarithm of initial reaction rates (ln v_i) on the reciprocal value of temperature ($1/T$), the activation energy (Ea) of 15 kJ/mol was calculated.

For the kinetic investigation of casein hydrolysis, a Lineweaver-Burk plot was constructed (Fig. 5). As can be seen in Fig. 5, casein hydrolysis obeys the Michaelis-Menten kinetics. From the slope and intercept with $1/v_i$-axis of the fitted straight line the Michaelis constant (K_M) of $1.35 \cdot 10^{-4}$ mol/dm^3 and maximal velocity ($v_{max.}$) of 3.37 mol/(dm^3·min) were calculated.



F i g . 5 – *Lineweaver-Burk plot for the enzyme catalyzed hydrolysis of casein performed at atmospheric pressure*

Proteinase-catalyzed hydrolysis of casein in SC CO_2

The same hydrolysis as was performed at atmospheric pressure was also carried out in SC CO_2 to optimize the pressure and temperature. Usually it is impossible to predict the stability of an enzyme in SC CO_2.[7] The number of parameters such as: temperature, pressure, water activity, pH etc., influence the stability of the enzymes in SCFs. The influence of the system pressure on the stability of enzymes is usually not so significant for pressure range up to 300 bar, but the temperature influences enzyme activity much more than pressure.

A small change in pressure is accompanied by a dramatic change in density, thus altering the density-dependent properties (partition coefficient, dielectric constant, Hildebrandt solubility parameter) of the SCF. Therefore the reaction rates may change with these physical parameters (this effect can be especially drastic near the critical point).[6,12,14]

Apart from the direct conformational changes in enzymes, which may occur at very high pressures, there is another way the pressure affects enzymatic reaction rates in SCFs. The *Eyring transition state theory* is used to explain the direct effect of pressure on the rates of reactions in SCFs:[14]

$$k = r \cdot \left(\frac{k_B \cdot T}{h} \right) \cdot K^*$$

where k is the reaction rate constant, k_B the Boltzmann constant (in J/K), h the Planck's constant (in J/s), T the temperature (in K), r a pressure and temperature independent coefficient, and K^* an equilibrium constant that is related to the difference in free energies between the transition state and the reactants.

Theoretically, the effect of pressure on reaction rate can be predictable if the reaction mechanism, activation volumes and compressibility factors are known. However, it is difficult to do this because the activation volumes of reaction steps and the compressibilities of SCFs change with pressure. A further complication is that, by changing pressure, one simultaneously changes the density-dependent physical parameters of the SCF. Effects of mass transfer are also always present to some extent. Therefore, only apparent activation volumes have been measured for enzymatic reactions in SCFs (also the exact reaction mechanisms of biocatalytical reactions are often not known).[6,12]

Effect of temperature and pressure

SCFs indicate unique combined properties such as liquid-like density and high compressibility, very low viscosity and high diffusivity. The first two properties make the solvent power controllable by changing pressure and/or temperature, while low viscosity and high diffusivity markedly enhance mass transport phenomena and hence kinetics of a process.

Changes in reaction rates could be reached if pressure and temperature are varied, because the transport properties of SCF may significantly change on one hand and on the other hand enzyme activation/deactivation may occur.[14] Combinations of pressure and temperature have influence on the solubility of substrates and products. Normally, higher solubilities of substances in SCFs are achieved with increase in temperature.[7] Although an increase of temperature enhances the rate of hydrolysis; the biocatalyst becomes more susceptible to inactivation.

Hydrolysis of casein in presence of proteinase from *Carica papaya* latex, which was also used for reaction performed at atmospheric pressure, was carried out at three different temperatures (35 °C, 40 °C and 50 °C) and three different pressures (100 bar, 200 bar and 300 bar) in SC CO_2. All other reaction parameters were the same as at reactions performed at atmospheric pressure. The reactions were performed in HP BSTR. The reaction mixtures were sampled after 5 minutes as well as after 4 hours of reaction.

Conversion after 4 hours decreased between 35 °C and 50 °C at 100 bar and 200 bar (Fig. 6). The highest degree of hydrolysis in SC CO_2 was observed at 200 bar and 35 °C. With increase in temperature, thermal deactivation of enzyme probably occurred also when the reaction was performed in SC CO_2. Increasing the pressure significantly affected the initial reaction rates, thus enabling final conversions to be obtained in shorter reaction

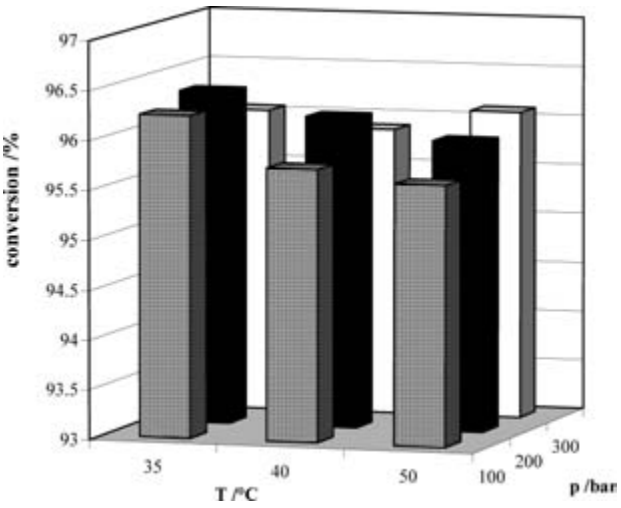


Fig. 6 – *Influence of pressure and temperature on equilibrium conversion of casein in SC CO_2*

times. By changing pressure, changes in density of CO_2 appeared, therefore also changes in physical parameters could be perceived. For that reason, the enzymatic reaction rates could also be changed. When the reaction was performed at 300 bar, the conversion achieved at 50 °C was similar to conversion at 35°C and 100 bar. At 300 bar and 50 °C the density of fluid was very high and therefore the increase in conversion was observed. Probably the impact of density power overcomes the deactivation of the enzyme which could occur owing to increase in temperature.

Density of SCFs is close to that of liquids; their viscosity is much lower in comparison with liquids. That results in higher diffusivity so they can speed up mass-transfer controlled reactions.

It is a well-known fact that raising of the temperature increases the reaction rate. Quantitatively the relationship between the reaction rate and reaction temperature was determined by the Arrhenius Equation.[15] Arrhenius plots were constructed for reactions performed at all three examined pressures (Fig. 7). For reactions performed at 100 bar the activation energy was calculated from data points obtained at all three investigated temperatures. However, for reactions performed at 200 bar only the data points at 35 °C and 40 °C were used in calculation. Although the positive values of activation energies were obtained, no significant differences in initial reaction rates were observed with increasing temperature. The data points obtained at 50 °C and 200 bar and 300 bar indicate that there were changes in the slope of Arrhenius plots; an additional mechanism had to be considered active in this temperature range – probably the deactivation of the enzyme.

The calculated activation energies (*E*a) for reactions performed at different pressures in SC CO_2 are presented in Table 2.

Table 2 – *Activation energies for enzyme-catalyzed casein hydrolysis performed in SC CO_2 at three different pressures (100 bar, 200 bar and 300 bar)*

	100 bar	200 bar	300 bar
Activation energy (*E*a) /(kJ/mol)	3.18	4.77	13.01

There are a number of mechanisms by which the activation energy decrease may be achieved. The most important of those involves the enzyme initially binding the substrate(s), in the correct orientation to react, close to the catalytic groups on the active enzyme complex and any other substrates. In this way the binding energy is used partially in order to reduce the contribution of the considerable activation entropy, due to the loss of the reactants translational and rotational entropy, towards the total activation energy.

Obviously, the influence of SC CO_2 also lowers activation energy. The activation energy increased with increase in pressure for enzyme catalyzed casein hydrolysis in SC CO_2, but the calculated values were lower than that obtained for the system at atmospheric pressure. The lowest activation energy appeared at pressure close to the critical one (100 bar).

Comparison of reactions performed at atmospheric pressure and in SC CO_2

Hydrolysis of casein, catalyzed by proteinase from *Carica Papaya* latex, was performed at atmospheric pressure at following optimal conditions: proteinase concentration of 10 g/L, casein concentration of 6.67 g/L, temperature of 30 °C and stirring rate of 600 rpm, while in the case when the reaction was performed in SC CO_2, optimal temperature was shifted to 40 °C and optimal pressure was found to be 200 bar. The course of both reactions is presented in Fig. 8.

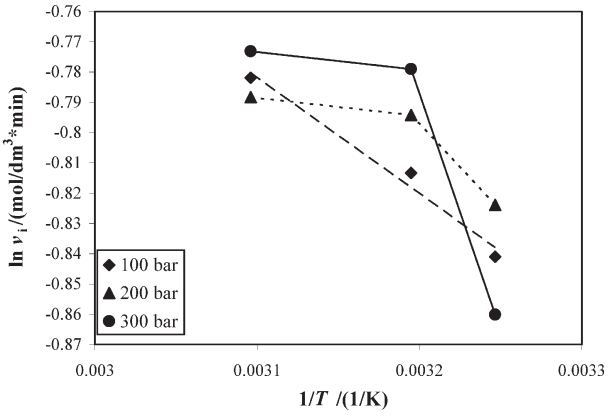


Fig. 7 – *Arrhenius plots for hydrolysis of casein in SC CO_2 catalyzed by proteinase from Carica papaya latex. Reactions at different temperatures and pressures were performed in the HP BSTR; (v_i – initial reaction rate)*

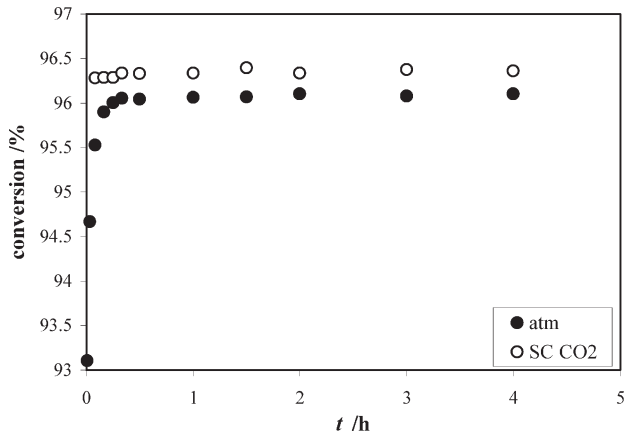


Fig. 8 – *Comparison of conversion vs. time profiles obtained for the enzyme-catalyzed casein hydrolysis performed at atmospheric pressure and in SC CO_2 at optimal conditions*

Reaction rate was improved when the reaction was performed in SC CO_2. The reason for the improvement in reaction rate are the transport properties of SC CO_2, resulting in lower mass-transfer limitations.

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