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Stability and catalytic properties of subtilisin in acetonitrile/dimethylformamide mixtures with low water content

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Abstract

Subtilisin 72 suspension retained high activity and stability in the triple mixtures acetonitrile/DMF/water (DMF concentration was up to 70% (v/v)). The synthetic activity of subtilisin suspension was investigated using the model reaction of tetrapeptide Z-Ala-Ala-Leu-Phe-*p*NA formation from Z-Ala-Ala-Leu-OMe and Phe-*p*NA ([S] = 30 mM, [E] = 6 μM; [S]/[E] molar ratio 5000:1). In the systems containing up to 60% DMF the 95% product yield was reached within 2 h. With DMF concentration increasing to 95%, the subtilisin catalytic efficiency notably decreased, though the product yield was still 30%. In the mixture acetonitrile/80%DMF/water, the effects of enzyme concentration, the reaction time and water content on the reaction progress were studied.

Keywords: Subtilisin; Low water media; Organic solvents; Enzymatic peptide synthesis

1. Introduction

Serine proteases and subtilisins, in particular, are widely used in organic synthesis for regio- and stereo-specific formation of amide and ester bonds in both aqueous and organic media [1–6]. The presence of organic solvents in the system shifts thermodynamic equilibrium toward formation of synthesis products and provides a high solubility of reagents, especially when long hydrophobic polypeptides are to be synthesized. In addition, some transformations in aqueous solutions are impossible because of secondary hydrolysis of synthesis products. Recent advances in nonaqueous enzymology revealed that many enzymes are effective catalysts in neat organic solvents. Yet, the catalytic activity of proteases in nonaqueous media is usually much lower than in aqueous media [3]. Such decrease is most pronounced in the system with polar solvents, such as DMF, DMSO, alcohols, etc. Replacement of water in protein microenvironment by organic solvent is believed to underlie this phenomenon [7]. As a result, the enzyme denatures and/or precipitates.

To improve the enzyme stability in organic media, various methods are used: alkylation with aldehydes [4,8], modification with polyethylene glycol [4,9], site-directed mutagenesis [4,10,11], the preparation of cross-linked crystals [4], lyophilization with additives of various nature [4,12,13], immobilization on various carriers [4,5] and noncovalent complex

Abbreviations: DMF: dimethylformamide; Z: benzyloxycarbonyl; *p*NA: *p*-nitroanilide

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formation of the enzyme with anionic detergents and polyelectrolytes [4,14].

The catalytic activity and stability of proteases are conventionally assayed by the examples of hydrolysis of their specific substrates [10,11]. However, this is difficult in the systems with high content of organic solvents. The catalytic activity in such systems is possible to define only after the aliquot has been transferred into water. The results thus obtained report rather about the enzyme capacity for reactivation, than its genuine properties in organic media.

Valuable results on the enzyme functioning in organic solvents can be obtained by studying the reaction of sugar esterification [4,8,9] and amino acid transesterification [12]. However, these data are insufficient for protease characterization, since this approach does not consider the main function of the proteolytic enzymes, the catalysis of reactions involving the peptide bond formation/hydrolysis. We believe it is the synthetic activity of proteolytic enzymes that enables one to most adequately describe the enzyme state in organic solvent systems since this parameter reflects the properties of the functioning enzyme. Until now peptide synthesis in these systems has largely been studied with protease suspensions [6], or proteases immobilized on solid carriers [4,5].

This paper reports the catalytic efficiency of the nonmodified enzyme, subtilisin, in media with a high content of polar organic solvents.

2. Experimental

2.1. Materials and methods

Solvents used: Acetonitrile was of HPLC purity grade from Lekbiofarm (Russia) and contained no more than 0.01% water, absolute DMF prepared as described earlier [15]. No special attempts were made to estimate water contents in these solvents. Serine proteinase from *Bacillus subtilis* strain 72 (subtilisin 72) was isolated and purified by method described elsewhere [16,17].

Reverse-phase HPLC was carried out on an Altex Model 100A liquid chromatograph (USA) using a Microsorb-MV C_8 column (4.6 mm × 250 mm; Rainin Instrument Company, USA). A linear gradient of acetonitrile from 20 to 100% in 35 min with 0.8 ml min^{-1} elution rate was used to elute the column. The eluate was monitored at 215 and 280 nm. No corrections were made to account for eventual differences between the molar extinction coefficients of the components.

Amino acid analyses were performed on a Hitachi-835 amino acid analyzer (Japan) after hydrolysis of the peptide samples with 5.7 M HCl for 48 h at 105°C. The results well agreed with those calculated for the product peptides.

2.2. Preparation of subtilisin suspension in acetonitrile / DMF mixtures

Lyophilized subtilisin 72 (1 mg, 35 nmol) was dissolved in 200 μl 0.05 M Tris–HCl buffer (pH 7.8) containing 1.5 mM $CaCl_2$, then 40 μl of this solution was added to 760 μl of the corresponding acetonitrile/DMF mixture (DMF concentrations in the resulting mixtures were 30, 40, 50, 60, 70, 80, 90 and 95%[1]) with stirring on a magnetic stirrer.

For studies with variable water content, the lyophilized subtilisin 72 (2 mg, 70 nmol) was dissolved in 114 μl 0.05 M Tris–HCl buffer (pH 7.8) containing 1.5 mM $CaCl_2$, then this solution was diluted to bring the final enzyme concentration to 6 μM; the resulting water content was 1, 2, 3, 4 and 5%. These enzyme solutions were added to 640 μl acetonitrile/DMF mixture with stirring on a magnetic stirrer. The DMF content was constant (80%), with decreasing acetonitrile content (19, 18, 17, 16 and 15%).

2.3. Determination of subtilisin activity suspension

Z-Ala-Ala-Leu-*p*NA solution 0.5 ml, 0.5 mg/ml in DMF was added to 2 ml 0.05 M Tris buffer (pH 8.2). The mixture was incubated for 10 min at 37°C. An aliquot (100 μl) of the enzyme suspension in acetonitrile/DMF mixture was added to initiate the reaction. The sample was incubated at 37°C until the yellow color appeared. The reaction was stopped by

[1] Hereinafter v/v % are given.

1 ml 1 M citric acid and A_{410} was measured. In the control sample, the enzyme solution was added after the reaction has been stopped. The specific activity was determined as

$$\text{Specific activity} = \frac{(A_{410} - A^{c}_{410})V^{s}}{A_{280}tV^{e} \times 8200},$$

where A_{410} is the absorption of the solution at 410 nm, A_{280} the absorption of the enzyme supernatant at 280 nm, A^{c}_{410} the absorption of the control sample, V^{s} the volume of the sample (ml), t the reaction time (min), V^{e} the volume of the enzyme solution and 8200 the molar extinction coefficient of the substrate ($M^{-1}\,cm^{-1}$).

2.4. Synthesis of Z-Ala-Ala-Leu-Phe-pNA in 65% acetonitrile / 30% DMF / 5% water mixture

To the solution of Z-Ala-Ala-Leu-OMe (5.1 mg, 12 μmol) and Phe-*p*NA (3.4 mg, 12 μmol) in the mixture of 120 μl dry DMF and 260 μl acetonitrile, 20 μl subtilisin solution in 0.05 M Tris–HCl buffer (pH 7.8) containing 1.5 mM $CaCl_2$ was added. The reaction mixture was stirred at 20°C, with 10 μl samples being periodically taken for HPLC-analysis.

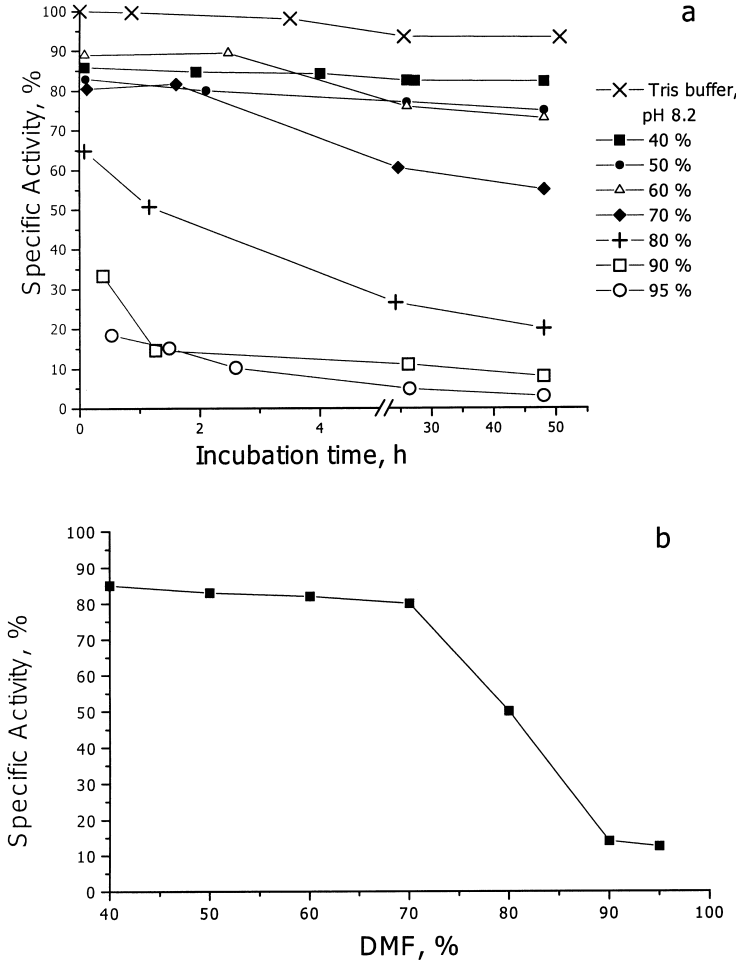

Fig. 1. (a) Stability of subtilisin in the mixtures DMF/CH_3CN/5%H_2O with various DMF content: Tris buffer, pH 8.2 (×); 40% (■); 50% (●); 60% (△); 70% (♦); 80% (+); 90% (□) and 95% (○). (b) The dependence of subtilisin specific activity on DMF concentration after 2 h incubation of the enzyme in the mixture DMF/CH_3CN/5%H_2O.

The reactions in the mixtures with 60, 80, 90 and 95% DMF were performed similarly.

Studies of the yield dependence on the enzyme and water concentrations were carried out similarly. In the studies of the yield dependence on the enzyme concentration, the resulting enzyme concentration was 0.3, 3, 6, 9 and 18 μM. In the studies of the yield dependence on water concentrations, the resulting enzyme concentration was 6 μM, the final water content was 1, 5, 7, 10 and 15%.

2.5. Preparative synthesis of Z-Ala-Ala-Leu-Phe-pNA

To the solution of Z-Ala-Ala-Leu-OMe (25.3 mg, 60 μmol) and Phe-*p*NA (17.1 mg, 60 μmol) in the mixture of 1.6 ml DMF and 0.3 ml acetonitrile, 100 μl subtilisin solution in 0.05 M Tris–HCl buffer (pH 7.8) containing 1.5 mM $CaCl_2$ was added. The reaction mixture was agitated on a magnetic stirrer for 24 h at 20°C. An undissolved protein was removed by centrifugation for 10 min at 16,000 g. The supernatant was added dropwise at gentle stirring to 12 ml 0.5 M HCl. The resulting precipitate was centrifuged, washed with water and dried in vacuo over NaOH. Then the dried substance was dissolved in 400 μl DMF and the procedure of precipitation in 0.5 N HCl was repeated. The resulting precipitate was centrifuged, washed with water and dried in vacuo over NaOH to yield 30 mg (73%) of the product.

Amino acid composition (nmol): Ala (10.4); Leu (5.4); Phe (5.2). HPLC retention time is 31 min.

3. Results and discussion

The object of our research, subtilisin 72, belongs to the class of serine proteases and is produced by the microorganism *B. subtilis* [16]. We studied the behavior of subtilisin 72 in the triple mixtures acetonitrile/DMF/water with low water content and various ratios of organic solvents. Water concentration was kept equal to 5% if not indicated otherwise, DMF concentration was varied in the range 30–95%, with acetonitrile concentration 0–65%. DMF was selected as the system component since it provides for high solubility of hydrophobic and hydrophilic amino acids, peptides and their derivatives. Acetonitrile was selected because it is widely used as solvent in peptide synthesis catalyzed by suspended and the solid support-adsorbed subtilisins [5,6]. Likewise, according to X-ray analysis, the conformations of subtilisin Carlsberg in acetonitrile and water are nearly the same [18]. The selected water concentration (5%) is typical for biocatalytic systems with polar organic solvents.

Subtilisin was introduced into the mixture of organic solvents in the form of its aqueous solution in 0.05 M Tris–HCl buffer (pH 7.8) containing 1.5 mM $CaCl_2$ with constant stirring. The presence of Ca^{2+}

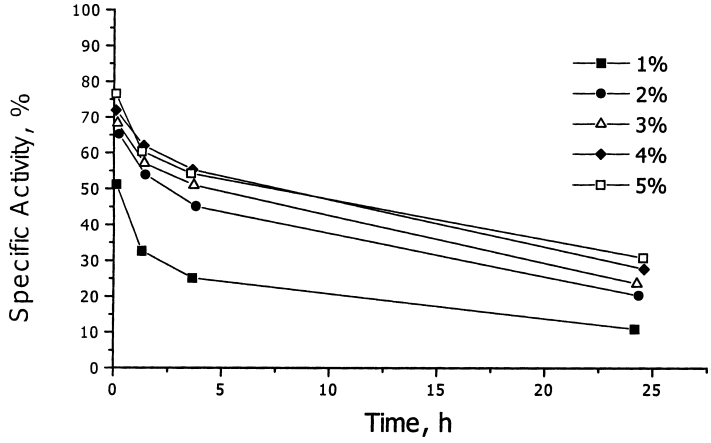

Fig. 2. Subtilisin specific activity after incubation in mixtures 80%DMF/CH_3CN/H_2O with various water content: 1% (■); 2% (●); 3% (△); 4% (♦) and 5% (□).

ions in buffer is essential for subtilisin stability. In the triple system (acetonitrile/DMF/water), subtilisin formed a suspension, which was confirmed by centrifugation. The dissolved subtilisin was not quantified, but solubility of subtilisin in DMF is known to be very low (about 6 ng/ml) [13]. We believe that the introduction of the enzyme into organic solvent in the form of its aqueous solution has some advantages compared to the suspending of lyophilized proteases (for instance, see [19]). According to Wong's data [13], the suspending of the enzyme powder in organic solvents leads to inaccessibility of a part of enzyme for interaction with substrate and to diffusion limitations.

The activity of the enzyme was estimated by the hydrolysis of its specific chromogenic substrate Z-Ala-Ala-Leu-*p*Na. To do this, an aliquot of the enzyme suspension in organic solvents was placed into 0.05 M Tris–HCl buffer solution (pH 7.8). The catalytic activity of subtilisin (which was not exposed to organic solvents) in aqueous buffer solution (pH 8.2) was taken as 100% relative activity. The results show that subtilisin retained the high activity and stability in the systems acetonitrile/DMF/5% water containing up to 70% DMF (Fig. 1a and b). Increase in DMF concentration up to 95% led to low residual activity, which was nevertheless retained even after 48 h incubation in organic media. The results were

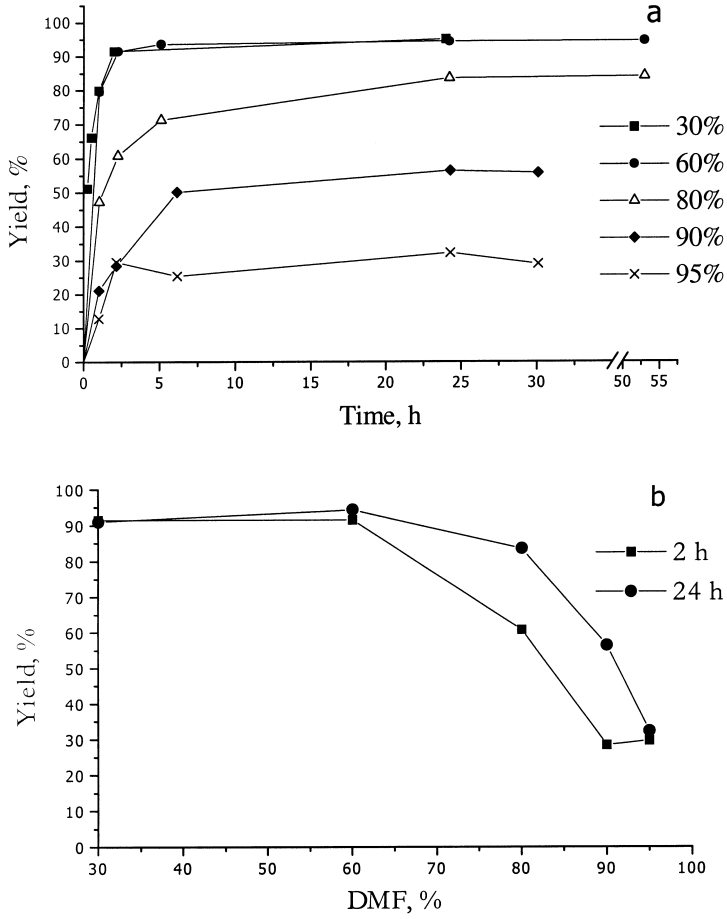

Fig. 3. (a) Dependence of the ZAALF*p*Na yield on the reaction time in the mixtures DMF/CH_3CN/5%H_2O with various DMF content: 30% (■); 60% (●); 80% (△); 90% (♦) and 95% (×). (b) The dependence of the ZAALF*p*NA yield on the DMF content in the reaction mixture: 2 h (■) and 24 h (●).

found to well agree with the data reported for subtilisin BPN' at 90 and 99% DMF [13]. Three ranges of DMF concentration (30–70, 70–90 and 90–95%) corresponding to various types of the enzyme behavior can be noted in the dependence presented in Fig. 1b.

The effect of water content on the enzyme hydrolytic activity after incubation in acetonitrile/80% DMF/water at the water content 1–5% was studied. The increase of water content from 1 to 5% little affected on the specific subtilisin activity. In all cases studied, the residual activity after 24 h incubation was about 20–40% (Fig. 2).

The synthetic activity of subtilisin in the triple mixtures acetonitrile/DMF/5% water with various ratios of organic solvents was investigated using the model reaction

$$\text{Z-Ala-Ala-Leu-OCH}_3 + \text{Phe-}p\text{NA} \xrightarrow{\text{subtilisin}} \text{Z-Ala-Ala-Leu-Phe-}p\text{NA} + \text{CH}_3\text{OH}$$

The reaction was carried out with equimolar amounts of amino- and acylating components ([S] = 30 mM) and at the [E] = 6 μM ([E]/[S] molar ratio 1:5000). The reaction progress was followed by using the reverse phase HPLC. Fig. 3a and b shows, that in the systems containing up to 60% DMF, the 95% product yield is reached rather fast (in about 2–3 h). These data correlate with subtilisin hydrolytic activity. With DMF concentration increasing from 60 to 95%, the maximal yield of the product decreased three-fold and the product accumulation stopped after 3–5 h reaction. Apparently, further product synthesis did not occur because of the enzyme inactivation. Thus, subtilisin catalytic activity in the systems with high DMF concentrations was low. Yet, there are indications of subtilisin-catalyzed reactions in nonaqueous polar solvents including transesterification and acylation of carbohydrates and other sugar-containing compounds in anhydrous DMF [20,21], and transesterification and peptide synthesis reactions in neat ethanol [22–24].

In this respect, it is of interest to compare subtilisin 72 synthetic activity in organic media with its hydrolytic activity after the transfer of the enzyme suspension in organic solvents into aqueous buffer solution. In the range of DMF concentration up to 70%, the data on both types of activity well correlate. At higher DMF concentrations, the estimations of the enzyme properties by synthetic and hydrolytic assays varied. The residual hydrolytic enzyme activity was observed even after 48 h incubation in 95% DMF whereas the enzyme was inactive to form the peptide bond in the same system after 3 h. Thus, in the mixtures with the high content of polar organic solvent (DMF) synthetic activity can be regarded as a more reliable criterion than the hydrolytic activity. Nevertheless, our results show that subtilisin can function in the mixtures containing up to 95% DMF.

The synthetic activity of subtilisin was studied in greater detail in the system acetonitrile/80%DMF/water since the product yield in this mixture is sufficiently high (Fig. 3a and b). Fig. 4 shows the dependence of Z-Ala-Ala-Leu-Phe-*p*NA yield on the

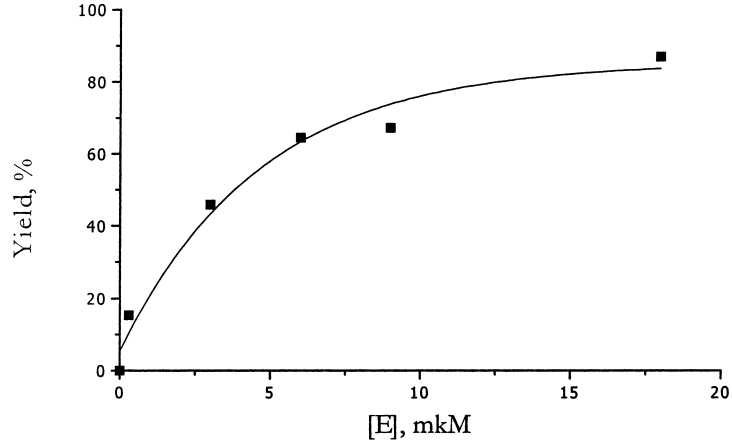

Fig. 4. Dependence of the ZAALF*p*Na yield on the enzyme concentration in the mixture 80%DMF/15%CH_3CN/5%H_2O.

enzyme concentration. The product was quantified after 2 h reaction. At the enzyme concentration < 6 μM, the yield of the product was rather low (Fig. 4). For instance, at 0.3 μM enzyme concentration, the yield was only 15% whereas at 6 μM, 65%. Further increase in the enzyme concentration did not affect the product yield.

The effect of water concentration in the mixture acetonitrile/80%DMF is shown in Fig. 5a and b. In the reaction mixture containing 1% water, the reaction ran rather slowly. The yield was 58% in 1 h and 90% in 24 h. With increasing water concentration to 5%, the reaction rate increased. The product yield was 68% after 1 h reaction, and 90% after 24 h. In the mixtures with 5 and 7% water, the yields were almost equal. In the systems with 10 and 15% water, the condensation rate was the highest; the maximal yield (90%) was reached after 5 h reaction. Then the product content in the reaction mixture decreased because of the secondary hydrolysis of the product (according to HPLC data). Thus, the increase in water concentration resulted both in the synthesis acceleration and in a notable secondary hydrolysis of the final product. Hence, water content in the system used for synthesis catalyzed by suspended subtilisin may be varied within a rather wide range. For subtilisin, adsorbed on a support, water content should not exceed 4%, but for the effective catalysis, the corre-

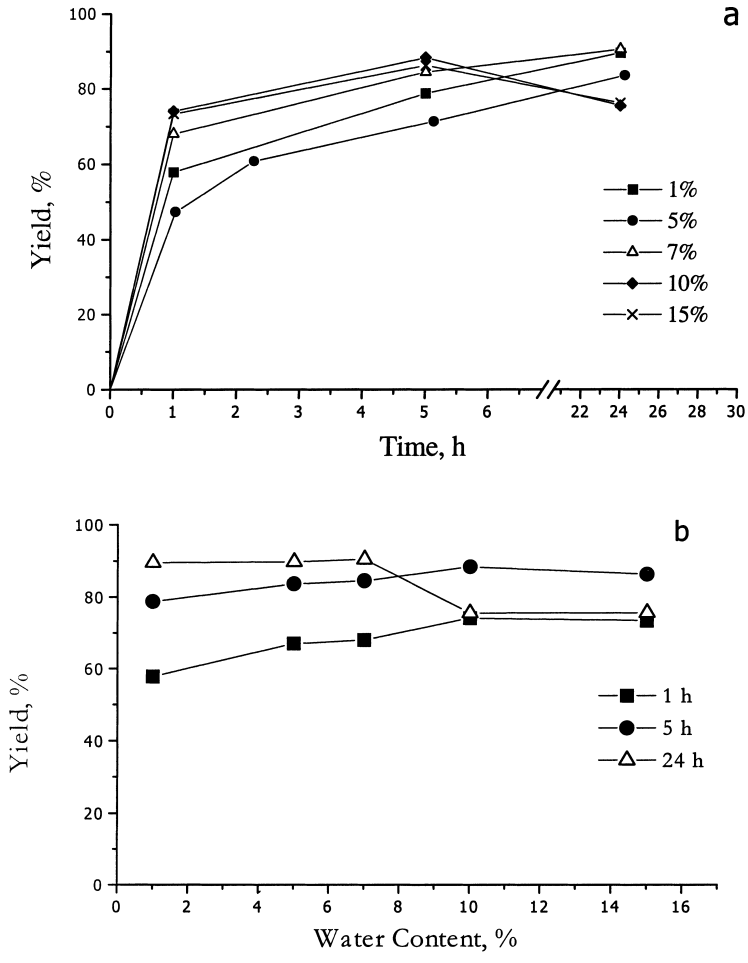

Fig. 5. (a) The time dependence of the ZAALF*p*NA yield in the mixtures 80%DMF/CH_3CN/H_2O with various water content: 1% (■); 5% (●); 7% (△); 10% (♦) and 15% (×). (b) The time dependence of the ZAALF*p*NA yield in the mixtures 80%DMF/CH_3CN/H_2O with various water content: 1 h (■); 5 h (●) and 24 h (△).

lation between water content and the reaction time should be allowed for minimize the effects of the side reactions.

Under the selected optimal conditions, we scaled-up the synthesis of Z-Ala-Ala-Leu-Phe-*p*NA. The reaction was catalyzed by subtilisin suspension and conducted in acetonitrile/DMF/5% water mixture. The reaction time was 24 h at [S]/[E] ratio 5000:1. In 24 h, the reaction mixture was centrifuged to remove the enzyme. Then, the product was isolated by precipitation from the supernatant with 0.5 H HCl and dried in vacuo. This isolation method provides for minimal losses of the final product. The product yield according to the described procedure was 73%. The product was characterized by HPLC and amino acid analysis data.

4. Conclusions

To conclude, in this work the behavior of subtilisin suspension in the triple mixtures acetonitrile/DMF/water was investigated. The enzyme activity and stability were studied by the example of substrate hydrolysis and peptide bond synthesis. Subtilisin was shown to retain the high activity and stability in the systems at up to 70% DMF concentrations. In the mixtures with high contents of organic solvents (more than 80%), the synthetic activity proved to be a more adequate criterion for catalytic efficiency of enzyme, than its hydrolytic activity.

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