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SDS–SUBTILISIN COMPLEX EFFICIENTLY CATALYZES SYNTHESIS OF PEPTIDES IN ETHANOL AND 2-PROPANOL

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Abstract: An enzymatic synthesis of tripeptide Z-Ala-Ala-Leu-pNA, tetrapeptides Z-Ala-Ala-P_1-P_1'-Xaa, where P_1 = Leu, Trp, Met, Ala, Ile, Phe; P_1' = Phe, Ala, Leu; Xaa = pNA, NH_2, pentapeptides Z-Ala-Ala-Leu-Ala-Ala-pNA and Z-Ala-Ala-Leu-Ala-Phe-pNA is described. The reactions were performed in organic solvents using SDS–subtilisin complex as a catalyst of the peptide bond synthesis. © 1997 Elsevier Science Ltd.

The use of proteases as catalysts of peptide synthesis is a promising direction of modern peptide chemistry. In a kinetically controlled process of peptide bond formation from peptide esters and aminocomponents one of the major obstacles is a secondary hydrolysis of the product. Peptide synthesis in organic solvents with low water content helps to solve this problem.

A new method of enzyme solubilization in polar organic solvents through formation of hydrophobic ion pairs has been reported recently.[1,2] It consists of preparation of the enzyme complex with an anionic detergent, for example, sodium dodecyl sulfate (SDS). It has been demonstrated that complexes of α-chymotrypsin and subtilisin BPN' with the detergent maintain the activity when solubilized in polar organic solvents.

The purpose of the present work was to study the possibility of using the complex of subtilisin 72 with SDS as a catalyst of peptide synthesis in alcohols.

Complex SDS–subtilisin was prepared by enzyme precipitation with SDS in the presence of Ca^{2+} ions,[1] then lyophilization and dissolution in an alcohol.

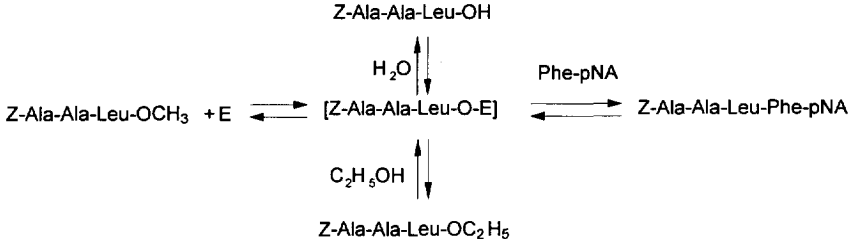
During the solubilization of SDS–subtilisin complex[1] in ethanol and 2-propanol, A_{280} gradually rose and achieved, after 1 h, a value of 0.5–0.6, then practically didn't change over a week. The UV-spectrum of subtilisin–SDS cqmplex solutions in alcohols did not differ from the UV-spectrum of the native subtilisin solution in water and revealed a maximum at 280 nm. The solubility of the complexes in 2-propanol and ethanol, evaluated on the basis of A_{280} values, attained 20 μM assuming that ε_{280} was the same in the alcohols and in water. Dissolution of the SDS–subtilisin complex in ethanol without preliminary lyophilization gave the same results. Native subtilisin 72 is only negligibly soluble in ethanol, which agrees with the literature data.[3]

Subtilisin, solubilised in the alcohols as the complex with SDS, preserves its potential activity for a long time. It was estimated via chromogenic substrate Z-Ala-Ala-Leu↓pNA hydrolysis after transfer of an aliquot of the enzyme alcohol solution to an aqueous buffer. The potential activity of SDS–subtilisin solution in ethanol decreased to 75% of the initial value during the first hour of incubation at 20 °C and then did not change for the next 48 h. After a week, the SDS–subtilisin solution in ethanol still preserved 60% of the original activity. The enzyme specific activity in 2-propanol decreased more rapidly to 60% during the first hour and maintained for the next 48 h. These results are in good accordance with the literature data[1] where the activity of the complex was assessed both by transesterification of Ac-Phe-OMe in alcohols or by the of Suc-Ala-Ala-Pro-Phe-pNA hydrolysis after the enzyme transfer to an aqueous solution.

To study an application of the SDS–subtilisin complex in enzymic peptide synthesis, we have chosen the following model reaction:

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

The structure of these components fits well to the specificity of subtilisin. The reaction was carried out with equimolar amounts of amino- and acylating components at $[E]/[S]$ $1{:}5{\cdot}10^3$ molar ratio. The course of the reaction was followed by the reverse-phase HPLC. Dependence of Z-Ala-Ala-Leu-Phe-pNA yield on the time and the enzyme concentration was studied at 20 °C. To ensure the solubility of the reactants, 20% (v/v) of DMF was introduced in all the reaction mixtures. The reaction passed according to the following scheme:



In 96% ethanol, the yield of Z-Ala-Ala-Leu-Phe-pNA after 30 min attained 56%, after 2 h –87%, then remained constant for 8 h. The yield of the product in dry ethanol attained 70% after 1 h. The reaction proceeded slower in 2-propanol, and the maximal yield (96%) was achieved only after 20 h.

When studying the dependence of the yield of a model peptide on the enzyme concentration (2 h, 96% ethanol) it has been demonstrated that below 5 μM concentration the yield decreased drastically, whereas at an enzyme concentration higher than 7 μM it reached a maximum value.

Under the conditions thus selected we prepared Z-Ala-Ala-Leu-Phe-pNA with the 82% yield. To isolate the peptide product its simple precipitation with water was used.

Essentially similar conditions were used for the synthesis of a series of N-protected amides and *p*-nitroanilides of tri-, tetra-, and pentapeptides (Table).

Table. Yields of peptides synthesized with SDS–subtilisin complex dissolved in ethanol[a]

Acylating component	Amino component	Product	Yield,[b] (%)
Z-Ala-Ala-Leu-OCH_3	H-Phe-pNA	Z-Ala-Ala-Leu-Phe-pNA	87(82)
Z-Ala-Ala-Leu-OCH_3	H-Leu-pNA	Z-Ala-Ala-Leu-Leu-pNA	98[c](80)
Z-Ala-Ala-Leu-OCH_3	H-Phe-NH_2	Z-Ala-Ala-Leu-Phe-NH_2	50[c](80)
Z-Ala-Ala-Leu-OCH_3	H-Leu-NH_2	Z-Ala-Ala-Leu-Leu-NH_2	50[c]
Z-Ala-Ala-Leu-OCH_3	HCl-Ala-Ala-pNA	Z-Ala-Ala-Leu-Ala-Ala-pNA	95
Z-Ala-Ala-Leu-OCH_3	HCl-Ala-Phe-pNA	Z-Ala-Ala-Leu-Ala-Phe-pNA	82
Z-Ala-Ala-Met-OCH_3	H-Phe-pNA	Z-Ala-Ala-Met-Phe-pNA	86
Z-Ala-Ala-Phe-OH	H-Phe-pNA	Z-Ala-Ala-Phe-Phe-pNA	48
Z-Ala-Ala-OCH_3	H-Leu-pNA	Z-Ala-Ala-Leu-pNA	50
Z-Ala-Ala-Trp-OCH_3	H-Phe-pNA	Z-Ala-Ala-Trp-Phe-pNA	33
Z-Ala-Ala-Ile-OCH_3	H-Phe-pNA	Z-Ala-Ala-Ile-Phe-pNA	0

[a]Condensation conditions: An acylating component, 31 mM; an amino component, 31 mM, solution of SDS–subtilisin in ethanol, 6 μM; 20 °C, 2 h. All received compounds were characterized by the data of HPLC and amino acid analysis of hydrolyzates.
[b]Preparative yields are given in parentheses.
[c]Reaction time 24 h.

Methyl esters of N-protected di- and tri- peptides and a N-protected tripeptide with free carboxyl group were chosen as acylating components. Leu, Trp, Met, Ala, Ile, Phe -amino acid residues occupied P_1- position in these components. As the amino components the amides and *p*-nitroanilides of amino acids and dipeptides were utilized, while Phe, Ala, Leu were present in P_1'- position. The use of Z-Ala-Ala-Ile-OCH_3 as the acylating component gave no product after 2.5 h, evidently on account of steric hindrances caused by isoleucine. This observation agrees well with the subtilisin specificity as revealed in the hexapeptides hydrolysis in aqueous solutions.[4] In all other cases satisfactory yields were achieved. Especially good were the yields of *p*-nitroanilides of N-protected peptides that usually approached quantitative ones even after 2 h. This conforms with numerous observations accomplished in our laboratory. *p*-Nitroanilide residue seems to be bound not only in S_2', but also

in S_3'- and perhaps in S_4'- subsite, promoting the reaction. Amides of tetrapeptides seems to be less favorable as nucleophiles and the yield did not exceed 50% in these reactions, even after longer time.

In conclusion, the specificity of subtilisin complexed with SDS seems to be comparable with that in water in what concerns peptide synthesis reaction. This study has demonstrated that the SDS–subtilisin complex can be successfully used for the enzymatic peptide synthesis.

Experimental

Materials and methods.

Solvents used: ethanol 96%, dry ethanol prepared by method,[5] 2-propanol of "chemically pure" grading, obtained from "Reachim" (Russia). 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.[6,7]

Reverse-phase HPLC was carried out on an Altex Model 100A liquid chromotograph (USA) using a Diasorb 130 C_8T column (4 × 250 mm; Biokhimmak, Moscow). A linear gradient of acetonitrile from 20 to 70% in 35 min with 1 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 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 at 105 °C for 48 h. The results were in good agreement with those calculated for the product peptides.

Preparation of SDS–subtilisin complex and its solutions in alcohols.

Lyophilized subtilisin 72 (2 mg, 70 nmol) was dissolved in 1 mL of 1mM $CaCl_2$, pH 5.5, then 500 μL of 7 mM SDS solution in water was added. The mixture was gently agitated, left at 20 °C for 1 h, then centrifuged for 15 min on Eppendorf Centrifuge 5415 C (Germany) at 16000 *g*. The supernatant was discarded, then 1 mL of an appropriate alcohol was added to the resulting precipitate and mixed for 1 h, then the mixture was centrifuged for 5 min at 16000 *g*. UV-spectrum of the supernatant and its A_{280} were measured.

The measuring of subtilisin and SDS–subtilisin complex activity.

To 0.5 mL of 0.5 mg/mL Z-Ala-Ala-Leu-pNA solution in DMF was added to 2 mL of 0.05 M Tris-buffer (pH 8.2) and the mixture was incubated at 37 °C for 10 min. An aliquot (10 μL) of the enzyme solution was added to initiate the reaction and the sample was incubated at 37 °C until the yellow coloration appeared. The reaction was stopped by the 1 mL of 1 M citric acid and the A_{410} was measured. In the negative control, the solution of the enzyme was added after the reaction was stopped.

*The analytical synthesis of **Z-Ala-Ala-Leu-Phe-pNA** in ethanol and 2-propanol.*

Z-Ala-Ala-Leu-OMe (24 mg, 57 μmol) and Phe-pNA (16 mg, 57 μmol) were dissolved in dry DMF (520 μL). To 50 μL of this solution alcohol (65 μL) and SDS–subtilisin complex solution in alcohol (60 μL, 0.55 mg/mL) were added. The mixture was agitated on a magnetic stirrer at 20 °C. Periodically 10 μL aliquots of the reaction mixture were taken for HPLC-analysis.

*The preparative synthesis of **Z-Ala-Ala-Leu-Phe-pNA** in ethanol.*

Z-Ala-Ala-Leu-OMe (21 mg, 50 μmol) and Phe-pNA (14 mg, 50 μmol) were dissolved in DMF (0.5 mL) and ethanol (0.8 mL). Then the SDS–subtilisin solution in ethanol (0.45 mL, 0.6 mg/mL) was added and the reaction mixture was agitated on a magnetic stirrer for 5 h at 20 °C. The solvent was evaporated in vacuo, then the dry yellow residue was treated with 0.5 mL of dry DMF. An undissolved protein was removed by the centrifugation for 7 min at 16000 *g*. The supernatant was added drop by drop under gentle mixing to 5 mL of water. The resulting precipitate was centrifuged off and dried in vacuo over NaOH that gave 28.6 mg of the product (82%).

Amino acid composition (nmol): Ala (10.4), Leu (5.4), Phe (5.2). HPLC retention time - 25.7 min.

Syntheses of the other peptides were performed similarly.

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8. Standard three letter abbreviations are used for amino acid residues, that all belong to L-series; pNA - *p*-nitroanilide.

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