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Synthesis of model peptide substrates and investigation of the reaction of their phenylacetyl protecting group enzyme transformation by means of penicillin G acylase

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The development of new protective groups by means of stereospecific and selective reactions is very important for the practice especially in the synthesis of semi-synthetic antibiotics and biologically active peptides. The aim of the present investigation is to study the kinetics of enzyme hydrolysis of the Phenylacetyl (Phac) group in synthetic peptide substrates. Two model compounds with two and three Phac, respectively, were studied. The enzymatic hydrolysis by means of penicillin G acylase in pH range 6–9 was studied. The substrate concentration was between 0.4–40 mM. Maximum degree of substrate conversion 75.5% (pH 9), which is closed to the degree of conversion to pH 7.8 (74.5%), was obtained for 3 to 10 min. At pH 6.5 and 7 the degree of hydrolysis was 62.5 and 37.5%, respectively. The possibilities for the reaction of hydrolysis in the presence of salts of different complex agents $CuSO_4.5H_2O$, $NiCl_2$, $Ni(NO_3)_2$ and $MnCl_2$ were investigated, too. There was 100% degree of Phac group hydrolysis in the presence of 10% $Ni(NO_3)_2$ for 9 min and full shift of equilibrium to the direction of hydrolysis was monitored in the presence of 25% $Ni(NO_3)_2$.

Key words: enzyme kinetic; penicillin G acylase; peptide mimetics; phenylacetyl protecting group.

INTRODUCTION

Peptides and α-amino acids are widely used in the medical practice. The development of new protective groups by means of stereo specific and selective reactions is very important for the practice especially in the synthesis of semi-synthetic antibiotics and biologically active peptides. Penicillin G acylase (PGA) is an enzyme that demonstrated high specificity on the phenyl acetyl residue. The enzyme's hydrolase activity is of a great importance for the new substrates modifications [1–5].

The aim of the present investigation is to study the kinetics of enzyme hydrolysis of the amide function between Phenylacetyl (Phac) group and amino acid residue.

EXPERIMENTAL

Materials and Methods

PGA [E.C 3.5.1.11] from *E. Coli* was supplied by Fluka (Switzerland). The enzyme activity of 980 E/ml was determined according to [6]. In the reaction solution, the product was diluted to 98 E/ml. The solution with concentration 0.15 mg/ml was used. KOH, KCl, NaOH, HCl; NH_4OH, KH_2PO4; K_2HPO_4 and CH_3CN - gradient grade for HPLC were received from MERCK (Darmstadt, Germany); $NiCl_2$; $Ni(NO_3)_2$; $CuSO_4.5H_2O$; $MnCl_2$ were purchased from Reanal (Budapest , Hungary)

The IR spectra were recorded on a Perkin-Elmer Model 1600 Series FTIR instrument. The purity of the products and kinetics of the hydrolysis reactions of phenylacetyl group were checked by RP-HPLC on a Perkin-Elmer apparatus, C_{18} column 4.6×250 mm ODS-A, S-5 micron 120 A. 50% 0.02 M KH_2PO_4:K_2HPO_4 pH = 7: 50% CH_3CN were used as eluents, rate 0.8 ml/min, λ = 220 nm and diode array detector.

The needed substrates Boc-Lys(Phac)-Val-OMe and Boc-Lys(Phac)-Lys(Phac)-Lys(Phac)-OH were obtained by conventional peptide synthesis in solution.

Boc-Lys(Phac)-Lys(Phac)-Lys(Phac)-OH was synthesized by drop-wise addition of Boc-Lys(Phac)-OH to C-terminal H-Lys(Phac)-OH by using of MA (mixed anhydrides) method:

1.00 mmol of Boc (or Z) amino acid (or peptide obtained from Boc-peptide by treatment with 10-fold excess of TFA) was dissolved in THF (10 ml) and after cooling to –10 ÷ –15°C and good stirring

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1.00 mmol Et_3N and 0.98 mmol isobutyl chloroformate were added. After 15 min 1.00 mmol of amino component without protecting groups dissolved in 1.1 mmol 2 N NaOH was added dropwise. The reaction mixture was stirred for 1 h at –10 ÷ –15°C and 24 h at room temperature. After that it was concentrated *in vacuo* and 2 N HCl was added to pH 2–3. The product was extracted with EtOAc (3×10 ml) and the organic layer was washed with 10% citric acid (3×10 ml) and H_2O to pH 7. The solvent was dried with Na_2SO_4 and removed *in vacuo*. The obtained products Boc-Lys(Phac)-Lys(Phac)-OH and Boc-Lys(Phac)-Lys(Phac)-Lys(Phac)-OH were oils.

Boc-Lys(Phac)-Val-OMe was obtained by the DCC/1-HOBt method:

1.00 mmol of HCl.H-Val-OMe was dissolved in DMF (10 ml) and after cooling to –5°C, neutralized to pH 7–7.5 with Et_3N. 1.2 mmoles of Boc-Lys(Phac)-OH; 1.20 mmol of DCC and 1.4 mmol of 1-HOBt were added. The reaction mixture was stirred for 24 h at room temperature. The obtained DC-urea was removed by filtration and then 30 ml of water were added. The product was extracted with EtOAc (3×10 ml) and the organic layer was washed with 5% $NaHCO_3$ (3×10 ml), H_2O (3×10 ml), 10% citric acid (3×10 ml) and H_2O to pH 7. The solvent was dried with Na_2SO_4 and removed *in vacuo* followed by recristalization in EtOAc/petroleum ether.

The obtained product has the following IR spectral characteristics: [cm^{-1}] 3328 ν_{N-H}; 3067 ν_{C-H} (aromatic system); 2930 ν_{C-H} (CH_3); 2853 ν_{C-H} (CH_2); 1740 $\nu_{C=O}$ (ester); 1700 $\nu_{C=O}$ (uretan); 1680 $\nu_{C=O}$ (amide I); 1661 $\nu_{C=O}$ (amide II); 1574 δ_{N-H} (amide I); 1545 δ_{N-H} (amide II); 1390 and 1367 δ_{C-H} (isopropyl and *tert*-butyl groups); 1247 ν_{C-O-C} (uretan); 743 $\delta_{C=C}$ and 696 $\delta_{C=C}$ (monosubstituted aromatic system).

General procedure for the reaction of hydrolysis of Phac by means of PGA

20 ml of substrate with the needed concentration in 10% DMF/0.1 M KCl were alkalized till pH 9 with 4 M K_2HPO_4. The solution of PGA with needed concentration was added. The hydrolysis of substrate was monitored on HPLC at 0, 1, 2, 3, 5, 9, 15, 30 and 60 min after acidification. During the reaction pH of the reaction mixture was hold up by titration with solution with pH 11–12 of complex agent's salt with needed concentration or NH_4OH.

RESULTS AND DISCUSSION

Two model reactions of hydrolysis of Phac were studied in the presence of substrates containing one and three Phac groups, respectively. The principal scheme of the reaction is presented in Figs. 1 and 2.

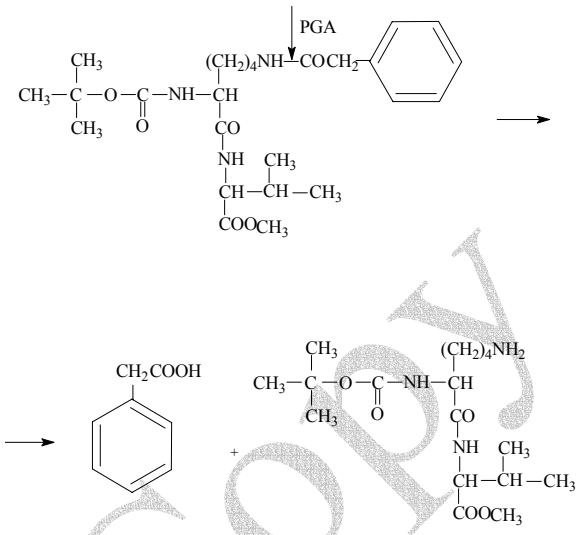

Fig. 1. Reaction of hydrolysis of Phac group in dipeptide ester Boc-Lys(Phac)-Val-OMe with PGA.

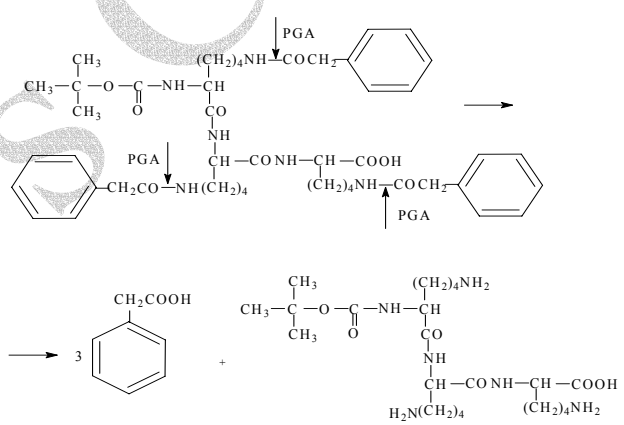

Fig. 2. Reaction of hydrolysis of Phac group in tripeptide Boc-Lys(Phac)-Lys(Phac)-Lys(Phac)-OH with PGA.

It is well known that pH optimum of PGA according to its natural substrate benzylpenicillin sodium or potassium salt is 7.8–8.0 [7–9]. In our investigation the used substrates are two peptides which are not specific substrates for PGA, thus the reaction of hydrolysis was monitored at pH range 6 ÷ 9. Series of reactions were carried out with 0.4 mM substrate at different pH (Fig. 3).

Maximum degree of substrate conversion 75.5% (pH 9), which is closed to the degree of conversion at pH 7.8 (74.5%), was obtained for 3 and 10 min, respectively. At pH 6.5 and 7, the degree of hydrolysis was 62.5 and 37.5%, respectively. By that reason, our next investigations on the reactions were carried out at the optimum value of pH 9 and at 37°C. Kinetic studies of the Phac group's hydrolysis were done with different enzyme concentrations in the synthetic substrate Boc-Lys(Phac)-Lys(Phac)-

Lys(Phac)-OH. The results are shown in Fig. 4. Maximum degree of substrate conversion 73.75% was obtained in the presence of 0.04 ml PGA for 8 min, which is closed to that obtained with 0.4 ml of PGA for 15 min (72.5%).

The obtained curves in the Figs. 3 and 4 clearly show that the reaction of Phac group hydrolysis is reversible. In order to attempt to shift the equilibrium of the reaction to the direction of full hydrolysis of the protecting group in the model peptide substrates and to make this reaction non-reversible, the same reaction was carried out in the presence of metal ions, which are good complexing agents. Their addition to the system might lead to the formation of complex with released ε-amino function of Lys residues (Fig. 5). The obtained complex might precipitate the forming product and to shift the reaction to the right direction. The possibility of similar complex formation was discussed previously by Schröder and Lubke [10].

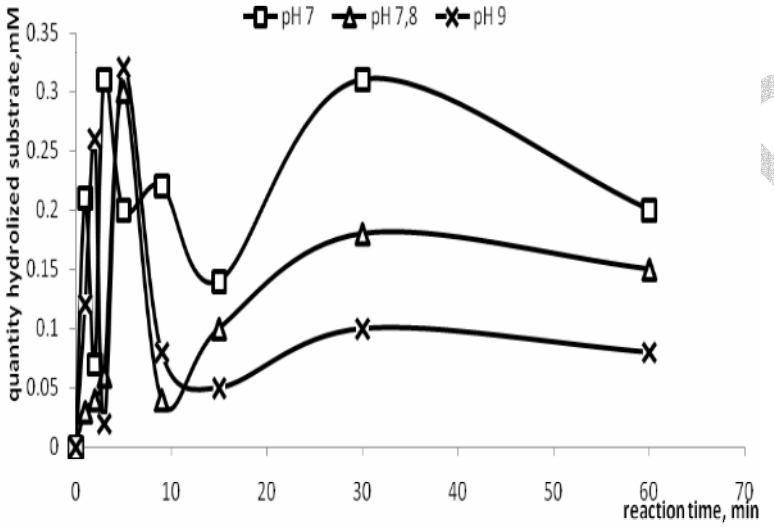

Fig. 3. Kinetics of hydrolysis of 0.4 mM synthetic substrate with 0.4 ml PGA at different pH.

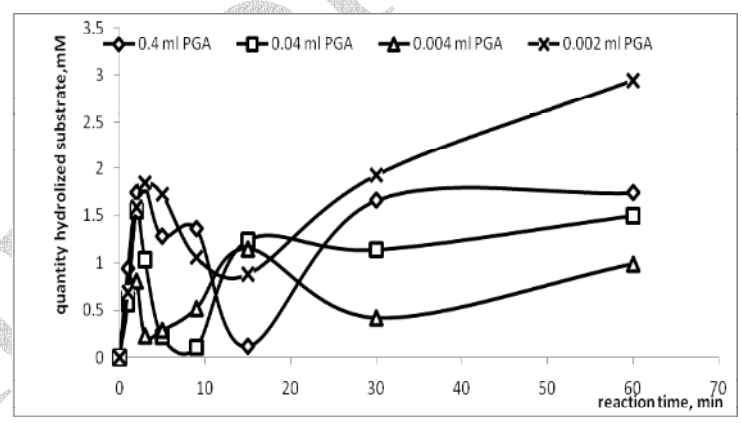

Fig. 4. Kinetics of hydrolysis of 5.0 mM synthetic substrate with different concentrations of PGA.

The kinetic results depending on the $Ni(NO_3)_2$ concentration, as a complex agent, are shown in Fig. 6. The figure clearly shows that the reaction equilibrium of hydrolysis of Phac group with stepwise addition of 10% $Ni(NO_3)_2$ was shifted to the direction of full hydrolysis of the protecting group for 9 min. In this case the substrate was 40 mM and the final concentration of $Ni(NO_3)_2$ – 1 M.

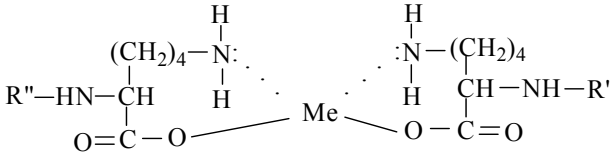

Fig. 5. Structure of the complex of ε-amino function of Lys residues with different metals.

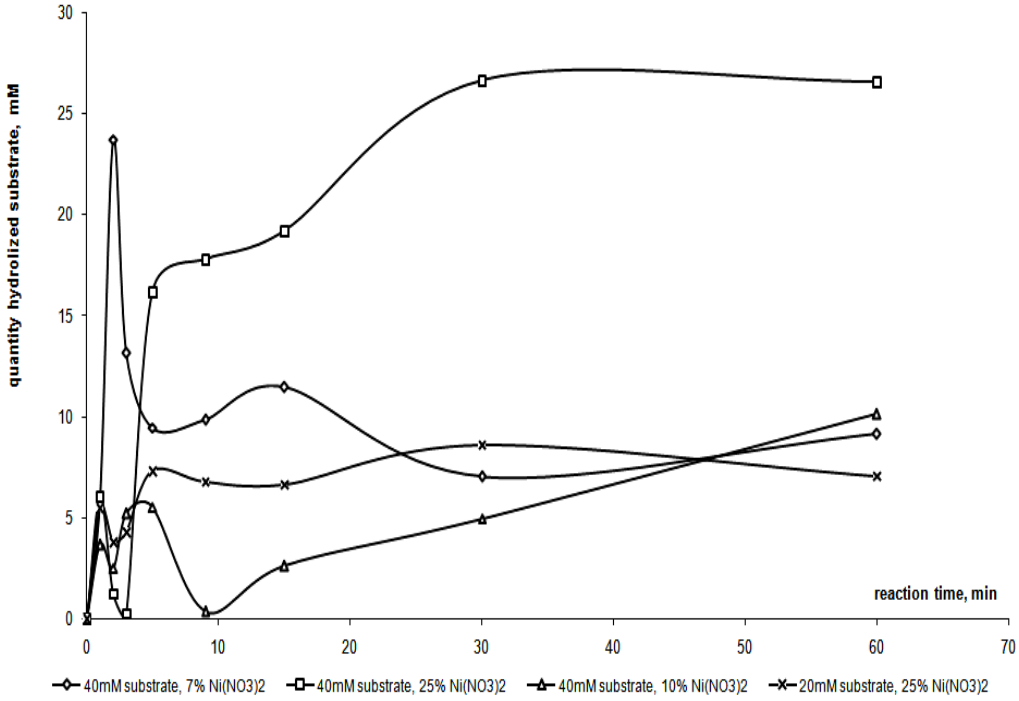

Fig. 6. Kinetics of hydrolysis of different amounts of synthetic substrate Boc-Lys(Phac)-Lys(Phac)- Lys(Phac)-OH with 0.002 ml PGA at pH = 9.0 and 37°C by stepwise addition of $Ni(NO_3)_2$ at different concentrations.

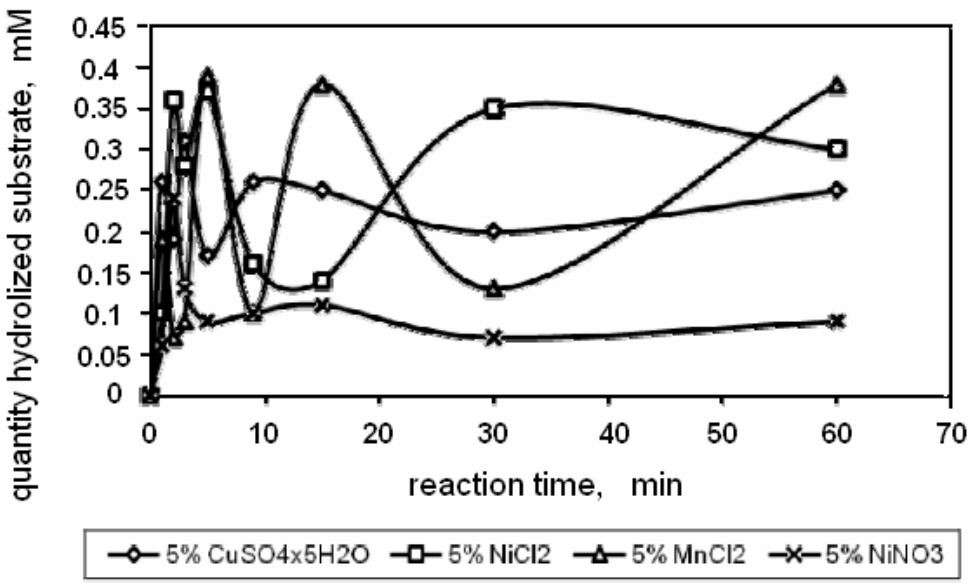

Fig. 7. Kinetics of hydrolysis of 0.4 mM synthetic substrate Boc-Lys(Phac)-Val-OH with 0.4 ml PGA in the reaction mixture at pH = 9.0 and 37°C by partial addition of different complex agents' salts.

The possibilities for the reaction of hydrolysis in the presence of salts of different complexing agents like $CuSO_4.5H_2O$, $NiCl_2$, and $MnCl_2$ were investigated, too (Fig. 7). As it can be clearly seen from the figure, the maximum degree of conversion was achieved in the presence of Ni^{2+} in the reaction mixture. The equilibrium of the reaction is shifted to the direction of full hydrolysis of the protecting group only in the case with stepwise addition of Ni^{2+}. It was revealed that the concentration of Ni^{2+} and the type of its salts are important, too. There was 100% degree of Phac group hydrolysis in the presence of 10% $Ni(NO_3)_2$ for 9 min and a full shift of the equilibrium to the direction of hydrolysis was monitored in the presence of 25% $Ni(NO_3)_2$.

CONCLUSIONS

The obtained results showed that the reaction of Phac group's hydrolysis was reversible at defined

conditions of pH, temperature range and reaction time.

Kinetic modelling studies of model peptide substrates reveal that:

- The use of metal ions as complexing agents of amino acid residues is a promising alternative for the studied enzymatic reaction.

- An acceptable approach is found to draw out the reaction of hydrolysis of phenylacetyl group with PGA in model peptide substrates to a desired direction.

- The best complex agents are Ni^{2+}.

- Maximum degree of substrate conversion (100%) was obtained at pH 9 and 37°C in the presence of 10% $Ni(NO_3)_2$.

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СИНТЕЗ НА МОДЕЛНИ ПЕПТИДНИ СУБСТРАТИ И ИЗСЛЕДВАНЕ НА РЕАКЦИЯТА НА ЕНЗИМНА ТРАНСФОРМАЦИЯ НА ФЕНИЛАЦЕТИЛНА ГРУПА С ПОМОЩТА НА ПЕНИЦИЛИН G АЦИЛАЗА

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(Резюме)

Откриването на нови защитни групи за органичния синтез, основани на стереоспецифични и стереоселективни реакции, е важно за практиката, особено при синтеза на полусинтетични антибиотици и биологично активни пептиди. Целта на настоящата работа е да изследваме кинетиката на ензимна хидролиза на фенилацетилна група в синтетични пептидни субстрати. Бяха използвани два моделни пептиди, съдържащи съответно една и три фенилацетилни защитни групи. Ензимната хидролиза беше направена с помощта на Пеницилин G ацилаза в широк pH диапазон 6–9. Използваните субстратни концентрации бяха 0.4–40 mM. Максимална степен на конверсия 75.5% (pH 9), близка до тази при pH 7.8 (74.5%) беше наблюдавана за 3 до 10 минути. При pH 6.5 и 7 степента на хидролиза беше съответно 62.5 и 37.5%. Беше изследвана възможността за провеждане на реакцията в присъствие на соли на различни метални комплексообразуватели $CuSO_4.5H_2O$, $NiCl_2$, $Ni(NO_3)_2$ и $MnCl_2$. Беше постигната 100% степен на конверсия на фенилацетилната група в присъствие на 10% $Ni(NO_3)_2$ за 9 минути и пълно изтегляне на химичното равновесие в посока на процеса на хидролиза в присъствие на 25% $Ni(NO_3)_2$.