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ENZYMATIC SYNTHESIS OF YIGSR - LAMININ PENTAPEPTIDE FRAGMENT- DERIVATIVES

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Abstract: An enzymatic synthesis of Ac-Tyr-Ile-Gly-Ser-Arg-OH, its amide and *p*-nitroanilide starting with Boc-Ile-Gly-Ser-OCH_3 is described. The reactions were performed in organic solvents using subtilisin and α-chymotrypsin sorbed on macroporous silica surface as the catalysts.

YIGSR - pentapeptide fragment of laminin - a basal membrane protein - is capable to bind, like laminin itself, to integrin on the surface of normal and tumor cells thus promoting their attachment to the basal membrane.[1] This peptide and its derivatives have been obtained by chemical methods, predominantly, by solid-phase peptide synthesis.[2] We developed an alternative approach using an enzymatic peptide synthesis to prepare N_α-acetylated YIGSR as well as two its derivatives - amide, earlier shown to be twice as active as YIGSR, and *p*-nitroanilide. The synthesis demonstrated the feasibility of enzymatic approach to form the peptide bond that only marginally fits to the specificity requirements of the enzyme used.

Initially, *p*-nitroanilide of the C-terminal tripeptide has been prepared by subtilisin-catalyzed condensation in organic solvents.

$$\text{Z-Gly-Ser-OCH}_3 + \text{H-Arg-pNA} \xrightarrow{\text{subtilisin}} \text{Z-Gly-Ser-Arg-pNA}$$

This reaction, although, proceeded very slowly notwithstanding twofold excess of the acylating component and gave only 23% yield of the product even after 96 h. Apparently, two factors hamper the reaction. Serine residue in P_1 position of the acylating peptide ester poorly suits to the specificity requirements of subtilisin S_1 substrate binding subsite[3] that favors hydrophobic side chains.[4] Moreover, only three of subtilisin substrate binding subsites - S_1, S_2 and S_3 - could be occupied by the N-protected dipeptide taking in account an eventual binding of benzyloxycarbonyl moiety at S_3 position.

Hence, this route was abandoned and another synthesis pattern was followed that envisaged arginine derivatives - amide or *p*-nitroanilide - acylation with Boc-protected tripeptide ester Boc-Ile-Gly-Ser-OCH_3 in organic solvents with minimal water content in the presence of sorbed subtilisin. Indeed, the synthesis of Boc-Ile-Gly-Ser-Arg-*p*NA catalyzed by subtilisin in 28% dimethylformamide solution in acetonitrile proceeded substantially better than H-Arg-*p*NA acylation with the dipeptide derivative. Molar ratio Boc-Ile-Gly-Ser-OCH_3 : H-Arg-*p*NA : subtilisin was $1:1.5:7.10^{-4}$. After 2 h

up to 98% of the amino component - H-Arg-*p*NA was converted into the tetrapeptide derivative as evidenced by HPLC. High yield simplified the isolation of the product that was precipitated with nonpolar solvent.

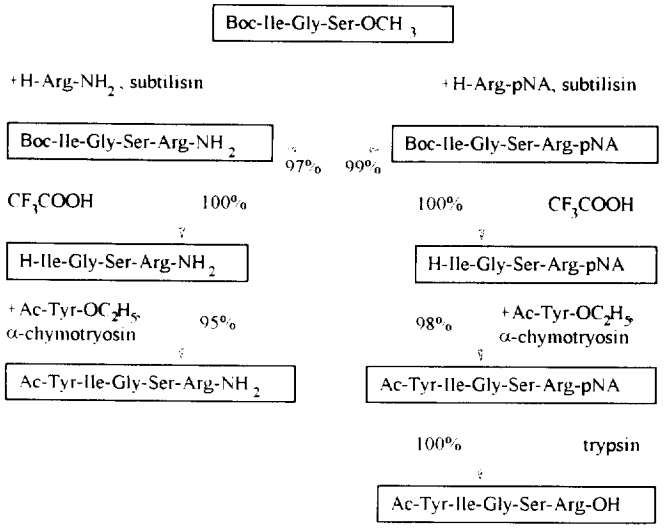
It appears that tripeptide derivative - Boc-Ile-Gly-Ser-OCH_3 satisfies better subtilisin specificity requirements than dipeptide Boc-Gly-Ser-OCH_3 due to occupation of four binding sites, including hydrophobic S_4 capable to interact with Boc group. This joint effect helps to overcome an unfavorable influence of hydrophilic serine residue binding at the S_1 area. Pancreatic elastase, thought to be more suitable than subtilisin for the acylation of the amino component with peptide derivatives that possess C-terminal serine, was also tested as a catalyst, but gave only 76% yield of the product. More difficulties were encountered in the course of the tetrapeptide amide synthesis. To dissolve an exceptionally hydrophilic arginine amide 25% dimethylsulfoxide solution in acetonitrile was used. Nevertheless, 97% yield was attained only after 10-15 days, whereas a fresh portion of sorbed enzyme was introduced into the mixture after 5-7 days. An excess of a less expensive component - arginine amide - was used. Hence, molar ratio of the reactants was as follows: Boc-Ile-Gly-Ser-OCH_3 : H-Arg-NH_2 : subtilisin $1:1.8:5.6\times10^{-4}$.

It seems that the process retardation ought to be explained by a difference in the binding of arginine *p*-nitroanilide and arginine amide by subtilisin. Whereas arginine amide acting as a nucleophile has to be bound at the S'_1 subsite known to accept arginine residues,[4] arginine *p*-nitroanilide that possess in addition an aromatic ring occupies presumably also the S'_2 subsite favorable for hydrophobic moieties, while *p*-nitro group might protrude even into the S'_3 subsite forming hydrogen bonds there. Eventual attempts to overcome this retardation *via* increase in arginine amide concentration might lead to incorporation of the second arginine residue into the product.[5]

After Boc-group removal with trifluoroacetic acid the tetrapeptide derivatives were converted respectively into the pentapeptide amide or *p*-nitroanilide *via* acylation of free α-amino group with fourfold excess of Ac-Tyr-OC_2H_5 catalyzed by α-chymotrypsin. The latter enzyme was preferred against subtilisin being substantially less sensitive towards the length of an acylating agent.[6] Molar ratio Ac-Tyr-OC_2H_5 : H-Ile-Gly-Ser-Arg-*p*NA : α-chymotrypsin corresponded to $4:1:3\times10^{-2}$. Notwithstanding highly increased amount of adsorbed α-chymotrypsin needed to compensate for too short an acylating agent, the condensation proceeded rather slowly in acetonitrile containing 18% dimethylsulfoxide, but gave almost quantitative (98%) yield of the products. Ac-Tyr-Ile-Gly-Ser-Arg-NH_2 was prepared analogously.

Trypsin hydrolysis of Ac-Tyr-Ile-Gly-Ser-Arg-*p*NA performed in water solution at pH 7.5 at substrate : enzyme molar ratio $1:4\times10^{-5}$ lead smoothly to formation of Ac-Tyr-Ile-Gly-Ser-Arg-OH. After removal of trypsin as a complex with soybean trypsin inhibitor and extraction of *p*-nitroaniline with ethyl acetate the acetylpentapeptide was isolated with almost quantitative yield.

Synthesis of YIGSR pentapeptide and its derivatives



EXPERIMENTAL

Starting peptides and derivatives have been prepared by conventional routes. α-Chymotrypsin purchased from Sigma and subtilisin 72 (an enzyme similar to subtilisin Carlsberg)[7] purified in this laboratory[8] have been distributed over macroporous silica - silochrom as described earlier [6]

Reverse-phase HPLC was performed on Gilson 704 chromatograph using Beckman Ultrasphere ODS column (4.6×250 mm). Solutions A and B contained 0.05% CF_3COOH and 0.05% $(C_2H_5)_3N$. Solution A - 5% CH_3CN, solution B - 90% CH_3CN. Detection was performed at 220. (peptide bond), 280 (Tyr) and 315 (pNA) nm.

Boc-Ile-Gly-Ser-Arg-*p*NA. To 817 mg (2.1 mmol) of Boc-Ile-Gly-Ser-OCH_3 and 525 mg (1.4 mmol) of H-Arg-*p*NA•HBr dissolved in 13 ml DMF 32.5 ml of CH_3CN was added followed by 30 mg (1 μmol) of subtilisin sorbed on 400 mg of silochrom. After agitation for 2 h at 20 °C, the catalyst was filtered off, the filtrate was concentrated *in vacuo* to 4 ml, then 100 ml of dry ethyl acetate was added. After 24 h at 4 °C the oily precipitate was treated with 30 ml of dry ethyl acetate, then crystallized under dry ethyl ether. Additional crop of the product was precipitated from the filtrate with toluene. Yield - 942 mg (92 %) of the tetrapeptide derivative. Amino acid composition (nmol): Ile (12.5), Gly (12.9), Ser (12.2), Arg (13.5). HPLC (gradient 30-60% of B buffer in 25 min) retention time - 14.1 min.

Ac-Tyr-Ile-Gly-Ser-Arg-*p*NA. To 300 mg (1084 μmol) of Ac-Tyr-OC_2H_5 and 200 mg (271 μmol) of Ile-Gly-Ser-Arg-*p*NA•CF_3COOH dissolved in 5.2 ml DMSO, 350 μl of 1M $(C_2H_5)_3N$ in CH_3CN and 22.6 ml of CH_3CN was added followed by 224 mg (10 μmol) of α-chymotrypsin sorbed on 1.5 g of silochrom. After agitation for 4 days the catalyst was filtered off and its fresh portion - 50 mg of α-chymotrypsin on 0.4 g of silochrom was added. After 24 h the catalyst was filtered off and washed, CH_3CN evaporated *in vacuo*, DMSO removed by lyophilization after sixfold dilution with water that gave 709 mg (85%) of the product. Amino acid composition (nmol) - Tyr (19.5), Ile (19.9), Ser (18.5), Arg (20.5). HPLC gave one peak with the retention time - 11.9 min (gradient - 25-35% of B buffer in 15 min).

Ac-Tyr-Ile-Gly-Ser-Arg-OH. 55 mg of Ac-Tyr-Ile-Gly-Ser-Arg-*p*NA were dissolved in 8 ml of water, pH 7.6 was adjusted with 0.1% NaOH. Under constant pH correction and stirring 0.1 ml of trypsin solution (1 mg/ml) was added. The mixture was kept for 15 min, then 100 μl of soybean trypsin inhibitor (1 mg/ml) was introduced into the solution. After 30 min the solution was ultrafiltered through Amicon YM-5 membrane and the filtrate lyophilized that gave 49 mg (89%) of Ac-Tyr-Ile-Gly-Ser-Arg-OH. Amino acid composition (nmol) - Tyr (15.4), Ile (15.2), Gly (15.5), Ser (14.9), Arg (16.0). HPLC retention time - 8.5 min (gradient of B buffer 10-25% in 15 min).

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9. Standard three or one letter abbreviations are used for amino acid residues, which belong to L-series; *p*NA - *p*-nitroanilide.

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