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V8 PROTEINASE-CATALYZED PEPTIDE SYNTHESIS IN HYDROPHILIC ORGANIC SOLVENTS WITH LOW WATER CONTENT

Václav Čeřovský

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, Czechoslovakia

Abstract: Free **Staphylococcus aureus** V8 proteinase was applied to coupling reactions of Z-Glu-OMe, Z-Glu-OBzl, Z-Ala-Glu-OMe and Z-Ala-Ala-Glu-OMe with various amino acid amides and peptides in acetonitrile containing 5 vol. % of water. No synthesis of Z-Asp-Leu-NH_2 was achieved under the same conditions. The synthesis of Z-Glu-Leu-NH_2 proceeded analogously also in 2-propanol or tert-butanol.

There are only few reports on applicability of highly specific Glu-C serine endoproteinase from **Staphylococcus aureus** strain V8 in peptide synthesis. Widmer et al.[1] synthesized sequences of mouse epidermal growth factor (EGF) by proteinase V8-catalyzed fragment condensation at Glu and Asp C-terminal residues (without experimental details). The S'-subsite specificity of V8 proteinase was studied by synthetic reactions using Z-Glu-OMe and a series of nucleophiles in the laboratory of Jakubke[2]. In the same laboratory V8 proteinase was successfully applied to the synthesis of model peptide[3] at -25 °C. Semisyntheses of carboxy-terminal fragments of thermolysin[4] were performed by the action of V8 proteinase in the presence of 50 vol. % of glycerol.

In our previous papers we reported peptide synthesis catalyzed by α-chymotrypsin[5], proteinase-K[6] and trypsin[7] in acetonitrile or aliphatic alcohols containing low amount of water. In all cases enzymes were used as aqueous solutions of commercial samples in the native forms without any modification or stabilization. Using the so-called kinetic approach we have proven that in these media serine proteinases catalyze the peptide bond formation in good yields depending on the character of the reaction

components (enzyme specificity). Reaction rate and yield can be influenced by the water amount in the system. At 5 vol.% of water concentration the acylating components were converted completely into peptides during one or two days and the undesired ester or secondary peptide hydrolysis was suppressed to minimum. Addition of organic base (triethylamine) was necessary to liberate free amine component from salt and to maintain basic conditions of the medium. Excess of triethylamine had no negative effect on the peptide yield. Similar results with slight discrepancies were independently published by Kise et al.[8].

In the present paper, aqueous solution of **Staphylococcus aureus** V8 proteinase was examined as catalyst for the synthesis of glutamic (aspartic) acid containing model peptides in acetonitrile, 2-propanol and tert-butanol. Empirically, the whole water content in the media was 5 vol.%, the amount of triethylamine was equimolar to hydrochloride (hydrobromide) salt of the nucleophile plus free side chain carboxyl of Glu (Asp). Considering high price of the enzyme its amount in the reactions was diminished fivefold as compared to enzyme amount used in our previous studies without any negative effect on the reaction rate or the peptide yield.

In order to provide useful predictive information for the use of V8 proteinase in peptide synthesis in hydrophilic organic solvents, the range of reaction components was tested under similar reaction conditions as shown in Table 1. Only slightly better result was obtained using Z-Glu-OBzl instead of Z-Glu-OMe as acylating component. Syntheses in alcohols were accompanied by higher extent of concurrent ester hydrolysis than using acetonitrile (no transesterification reaction was detected). In the case of lower water concentration (2 vol.%) no substantial effect on the reaction was observed. The peptides as acyl components appeared to be better substrates than Z-Glu-OMe. It has been reported[2] that in aqueous media V8 proteinase catalyses preferentially the formation of Glu-X peptide bonds, where X is a hydrophobic residue. In acetonitrile only Leu-NH_2 and Leu-Gly were acylated with satisfactory yields by the action of V8 proteinase. In the reactions with Ala-Ala and Leu-OBu^t as nucleophiles, only hydrolyses of Z-Glu-OMe were observed (5% yield of Z-Glu-Ala-Ala was achieved in aqueous medium[3]). On the other hand[2], the apparent synthesis of Z-Glu-Gly-NH_2 proceeded in acetonitrile.

No reaction was detected with Z-Asp-OMe as acyl component in this study. The successful syntheses of -Asp-X- peptide bonds in EGF using V8 proteinase in aqueous solution were described by Widmer[1], contrary to the facts that V8 proteinase hydrolysed -Asp-X- peptide bonds in the proteins[9] or peptides[10] only in very special instances.

Table 1. V8 proteinase-catalyzed synthesis of peptides in hydrophillic organic solvents containing 5 vol.% of water

Acyl component	Amine component	Peptide synthesis (%) 4 h	24 h	Ester hydrolsis (%) 4 h	24 h
Z-Glu-OMe [a]	Leu-NH_2.HCL	78	90	8	10
Z-Glu-OBzl [a]	Leu-NH_2.HCl	85	93	6	7
Z-Glu-OMe [b]	Leu-NH_2.HCl	68	73	25	27
Z-Glu-OMe [c]	Leu-NH_2.HCl	76	77	24	23
Z-Glu-OMe [d]	Leu-NH_2.HCl	77	94	5	6
Z-Glu-OMe [a]	Gly-NH_2.HBr	23	31	5	7
Z-Glu-OMe [a]	Leu-Gly	51	75	11	15
Z-Glu-OMe [a]	Phe-NH_2	49	49	30	51
Z-Glu-OMe [a]	Trp-NH_2.HCl	18	16	77	79
Z-Ala-Glu-OMe [a]	Leu-NH_2.HCl	92	92	8	8
Z-Ala-Ala-Glu-OMe [a]	Leu-NH_2.HCl	86	91	8	9
Z-Asp-OMe [a]	Leu-NH_2.HCl	0	0	0	0

[a] in acetonitrile, [b] in 2-propanol, [c] in tert-butanol, [d] in acetonitrile containing 2 vol.% of water

Experimental procedure

Staphylococcus aureus V8 proteinase (EC 3.4.21.19), lyophilized powder, activity 900 units/mg was SIGMA (U.S.A.) product. Z-Glu-OMe, Z-Asp-OMe, Z-Ala-Glu-OMe and Z-Ala-Ala-Glu-OMe were synthesized by standard procedures starting from Z-Glu(OBu^t)-OH and Z-Asp(OBu^t)-OH. Other compounds were either purchased from Serva (F.R.G.) or were available from our collection.

In a typical experiment, triethylamine (4.5 µl, 0.033 mmol) was added to a solution of Z-Glu-OMe (3 mg, 0.01 mmol) and Leu-NH_2.HCl (3.4 mg, 0.02 mmol) in acetonitrile or alcohol (95 µl). A solution of V8 proteinase (0.01 mg) in water (5 µl) was added and the mixture was incubated at 30 °C with constant shaking. In order to follow the time course of the reactions, at regular time intervals aliquots (2 µl) were withdrawn and injected into 50% aqueous methanol containing 1% of trifluoroacetic acid (100 µl) and then analyzed by HPLC. HPLC was performed on a Spectra Physics SP8700 instrument equipped with SP8400 UV detector, SP4100 integrator and Vydac C_{18} column (0.46 x 25 cm); flow rate 1 ml/min, detection at 254 nm, mobile phase methanol with 0.05% aqueous trifluoroacetic acid (50/50 by volume).

Products of syntheses were separated on the preparative Vydac C_{18} column (1 x 25 cm) using gradient elution with the same mobile phase at flow rate 3 ml/min.

Identities of isolated products were verified by amino acid analyses and by mass spectra: Z-Glu-Leu-NH_2 (Glu 1.00, Leu 0.96), $(M+H)^+$ 393; Z-Glu-Gly-NH_2 (Glu 1.00, Gly 0.98), $(M+H)^+$ 338; Z-Glu-Leu-Gly (Glu 1.00, Gly 0.99, Leu 1.00), $(M+H)^+$ 451; Z-Glu-Phe-NH_2 (Glu 1.00, Phe 0.97), $(M+H)^+$ 428; Z-Glu-Trp-NH_2 (Glu 1.00, Trp 0.82), $(M+H)^+$ 467; Z-Ala-Glu-Leu-NH_2 (Glu 1.00, Ala 1.00, Leu 0.97), $(M+H)^+$ 465; Z-Ala-Ala-Glu-Leu-NH_2 (Glu 1.00, Ala 1.89, Leu 0.99), $(M+H)^+$ 536.

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