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ENZYME PEPTIDE SYNTHESIS BY AN ITERATIVE PROCEDURE IN A NUCLEOPHILE POOL

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Summary: An effective method for enzyme solubility-controlled synthesis of peptides, consisting in an iterative addition of equivalent amounts of acyl and amine components to a solution(nucleophile pool) containing the enzyme and a large excess of the amine component, is described.

Proteinases are not only proteolytic but proteosynthetic enzyme as well[1,2]. Enzyme specificity suppresses the side reactions (side chain modification and racemization) and ensures the synthesis of chemically and chirally pure peptides. In solubility-controlled accumulation of the product[3], the peptide precipitates to keep its concentration below the equilibrium value. Usually, the resulting deposit is collected by filtration and the filtrate, containing the enzyme and the unreacted reagents, is discarded[4]. This implies that the enzyme synthesis can not be actually applied in the case of low nucleophile specificity which would require a large excess of amine component which would be discarded. The main problem, however, is recycling of the enzyme. An iterative use by immobilization on a solid support is complicated by the tedious separation of the immobilized enzyme from the deposition product[5]. Due to the slow diffusion of the reagents and decrease in enzyme activity, several times longer reaction time is needed in immobilized-enzyme synthesis in aqueous-organic solvent mixtures[5,6]. Here, we report an effective method for enzyme peptide synthesis, consisting in an iterative addition of equivalent amounts of acyl and amine components to a solution(nucleophile pool) containing the enzyme and a large excess of the amine component, followed by filtration of the product.

Enzyme hydrolysis or synthesis proceeds through the formation of an acylenzyme that is hydrolysed by water W or aminolysed by an amine component N[2]. If k_w and k_n are the rate constants of these parallel reactions, the preparative yield Y in the synthetic reaction is given by the following expression:

$$Y = \frac{100k_n[N]}{k_n[N] + k_w[W]} \% \qquad 1.$$

Therefore, a prerequisited yield Y_p can be obtained using a nucleophile concentration

$$[N] = \frac{55Y_p}{(100-Y_p)(k_n/k_w)} , \qquad 2.$$

i.e. it depends on the aminolysis/hydrolysis ratio k_n/k_w. Being a measure of the enzyme nucleophile specificity, this ratio varies considerably[7]. From eq.2 follows that in the case of low nucleophile specificity(low k_n/k_w-value) a high preparative yield could be obtained using high nucleophile concentrations. If the reaction is carried out in such a nucleophile pool, a simple filtration of the deposition product makes the pool ready for another run. Actual ly, the enzyme synthesis can be carried out again by simple addition of equivalent amounts of the acyl and amine components into the pool. By iteration of this procedure, the peptide synthesis in the case of low nucleophile specificity and recycling of the enzyme can be achieved.

TABLE I

Chymotrypsin Synthesis of Z-Tyr-D-Leu-NH_2 in a 10 ml Nucleophile Pool, Containing 0.01 M H-D-Leu-NH_2, 0.001 mM α-Chymotrypsin and 0.002 M Na_2CO_3, pH 9.3, 25°C.

Iteration	Reagents Added (M)		Reaction Time (min)	Yield (%)
	Z-Tyr-OMe	H-D-Leu-NH_2		
basic	0.002	0.010	45	64
I	"	0.002	45	85
II	"	"	45	85
III	"	"	50	90
IV	"	"	60	90
V	"	"	65	90

H-D-Leu-NH_2 is a poor nucleophile in chymotrypsin peptide synthesis. Its k_n/k_w-value, determined as described earlier[7], is about 8 times lower than that of the corresponding enantiomer H-Leu-NH_2. This suggests that the concentration of the nucleophile should be 8 times higher for the same preparative yield. The pool was prepared by dissolving 1.65 g(0.01 M)H-D-Leu-NH_2.HCl[8] and 24 mg(0.001 M) α-chymotrypsin(Reachim-USSR) in 10 mL 0.2 M carbonate buffer pH 9.3 (Table I). Under vigorous stirring 0.65 g (0.002 M) solid Z-Tyr-OMe[9] was added. In 15 min the suspension thickend and 30 min later no trace of the acyl component was found as judged by t.l.c. analysis. The suspension was filtered to remove the product. The first iteration was carried out by addition of 0.33 g (0.002 M) H-D-Leu-NH_2.HCl in the filtered nucleophile pool followed by suspending 0.65 g (0.002 M) Z-Tyr-OMe. After the

conversion of the acyl component was checked by t.l.c., the nucleophile pool was made ready for the second iteration by filtration of the product.

After 5 iterations the products were pooled, washed with buffer and dried; total yield 85 % (4.8 g) based on Z-Tyr-OMe and 43% (being 17% in one run) based on H-D-Leu-NH_2. The yield is lower in the basic iteration(Table I) due to the lack of the product saturation in the nucleophile pool. After recry stallization from methanol/water, the peptide was found homogeneous by t.l.c. (solvent system $CHCl_3/CH_3COOH$/petroleum ether 3.0:0.5:0.5); m.p. 110-112°C; $[\alpha]_D^{20}$=46.4(c 0.4 MeOH); NMR[10] (Me_2SO-d_6) δ 4.2 (1H, Tyr αH), 3.8 and 3.68 (2H Tyr βH), 6.65 and 7.05 (4H, Tyr Ar H), 5.0 (2H, Z CH_2), 0.81 (6H, Leu δH), no signals for D-Tyr and Leu were observed. Calcd. for $C_{23}H_{29}N_3O_5$, C, 64.63; H, 6.79; N, 9.84; found C, 64,44; H, 6.79; N, 10.03.

The chymotrypsin synthesis of Z-Tyr-D-Leu-NH_2 described above demonstrates an effective method for enzyme incorporation of D-amino acids into the amino acid sequence of peptides. Substitution of D- for the natural L-amino acids plays an important role in the design of stable against enzyme degradation biologically-active peptides. A chemical-enzymatic synthesis of [D-Ala^2, D-Leu^5]enkephalinamide and the corresponding diastereomers will be described separately.

TABLE II

Thermolysin Synthesis of Z-Asp-Phe-OMe (Z-Aspartame) in a 10 mL Nucleophile Pool, Containing 0.008 M H-Phe-OMe, 10 mg Thermolysin, pH 6.8, 40°C.

Iteration	Reagents Added (M)		Reaction Time (hour)	Yield (%)
	Z-Asp-OH	H-Phe-OMe		
basic	0.002	0.008	3	88
I	"	0.004	3	96
II	"	"	3	88
III	"	"	3.5	96
IV	"	"	4	96

Unlike chymotrypsin, thermolysin shows its primary specificity to the amine component[11]. As has been shown by Isowa et al.[12], in a thermodynamically-controlled thermolysin synthesis of Z-Asp-Phe-OMe, Z-Asp-OH and H-Phe-OMe reacted in a 1:2 ratio, the second equivalent of the amine component being necessary for the formation of the deposition product - a salt of the peptide with H-Phe-OMe. An excess of the amine component, however, provides shorter reaction times. Thus a nucleophile pool was prepared (Table II) by dissolving

1.76 g (0.008 M) H-Phe-OMe.HCl[13] and 10 mg thermolysin (Boehringer) in 10 mL water. 0.53 g (0.002 M) Z-Asp-OH[14] was than added and the enzyme synthesis was initiated by adjusting the pH to 6.8. After the conversion of the acyl component the resulting deposit was filtered to make ready the nucleophile pool for the first iteration.

The products from all iterations were collected, washed with buffer and dried; total yield 5.6 g (93%). Recrystallized free Z-Asp-Phe-OMe melts in the interval 120-4°C (lit.[15] 118-124); $[\alpha]_D^{25}$= -14.4 (c 1 MeOH)(lit.[15] -14.6) and is homogeneous by t.l.c.(solvent system $CHCl_3/CH_3COOH$/petroleum ether 3.7:0.3:1.0) NMR[10] (Me_2SO-d_6) δ 3.62(3H, OCH_3), 5.04(2H, Z CH_2), 7.54(1H, Asp NH), 8.28 (1H, Phe NH), no signals for the corresponding epimers were observed.

Z-Asp-Phe-OMe is a precursor of the peptide sweetener aspartame. The latter is 200 times sweeter than sucrose[16]. Being the only artificial sweetener that does not carry a health warning concerning cancer, its widespread use in food products and beverages is in prospect[17]. The method for its enzyme synthesis by the iterative procedure in a nucleophile pool is alternative to the method using an immobilized enzyme.

In both examples of the method we described, the procedure was iterated several times, thus recycling the enzyme several times. For further iterations a correction of the concentration and volume of the nucleophile pool is necessary, since part of it remains bound to the wet product.

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