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ENZYMIC SYNTHESIS OF OLIGOPEPTIDE—VI

THE MECHANISTIC FEATURES OF PEPSIN-CATALYSED PEPTIDE SYNTHESIS

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Abstract—The dipeptide Z-Phe-Phe-OBzl and tripeptide Z-Phe-Phe-Phe-OBzl were synthesized by pepsin catalysis from the incubation of Z-Phe and Phe-OBzl in the reaction solution. The yield ratio of two peptides in relation to reaction time was investigated by HPLC. Another example: The tripeptide Z-Phe-Leu-Phe-OBzl and tetrapeptide Z-Phe-Leu-Phe-Phe-OBzl were also synthesized concurrently from Z-Phe-Leu and Phe-OBzl by pepsin catalysis. These results may have important implication for the transpeptidation of pepsin. But, according to the report of Pellegrini and Luisi, the dipeptide, Z-Phe-Phe-OBzl, synthesized by pepsin catalysis was not contaminated with the tripeptide, Z-Phe-Phe-Phe-OBzl, and the yield was high. In order to investigate the discrepancies between our observation and those reported by Pellegrini and Luisi, a mechanism by which pepsin synthesizes the dipeptide, Z-Phe-Phe-OBzl, and tripeptide, Z-Phe-Phe-Phe-OBzl, is proposed and the supporting data demonstrated by HPLC analysis.

The use of proteolytic enzymes to catalyse peptide bond formation may become one of the synthetic methods of the future.[1] The principle involved depends on shifting the chemical equilibrium toward peptide bond formation, by employing suitable protecting groups, which yield an insoluble product.[2] The enzymic synthesis proceeds at room temperature in buffered aqueous-organic solution, and such a mild condition should minimize or eliminate the possibility of a side reaction in conventional chemical coupling. Racemization may always be expected to some extent in chemical coupling, with the enzyme's stereospecificity, the optical purity of the product should be the highest among any peptide bond formation reactions.

Despite these promising features, the enzymic procedure still has some drawbacks. When a proteolytic enzyme is used as a catalyst for peptide fragmental condensation, the hydrolytic action to the pre-existing, scissible bonds should be noted. If hydrolysis occurs, the resultant cleavage products may act as substrates and give rise to undesired compounds.[3]

It is known that a proteolytic enzyme can catalyse a transpeptidation reaction, and convert some peptide substrates to more complex product mixtures.[4,5] If transpeptidation occurs, the resultant products may also act as substrates and give rise to a complex product mixture.

Recently, we reported the transpeptidation reaction in a multiple step peptide bond formation by proteolytic enzymes.[3] When amino-protected amino acid was coupled to carboxyl-protected amino acid, the precipitated protected dipeptide was shown to contain substantial amounts of protected tripeptide depending on the enzyme used. Pellegrini and Luisi[2] have reported the synthesis of Z-Phe-Phe-OBzl by pepsin catalysis with high yield and without any contamination of tripeptide Z-Phe-Phe-Phe-OBzl. These results are quite different from ours. In this paper, we report a detailed description of the consecutive formation of dipeptide and tripeptide using amino-protected amino acid and carboxyl-protected amino acid as substrates in which the peptide bonds are prepared by pepsin mediated catalysis. Elucidation of the catalytic effect may provide information regarding the mechanism of pepsin action and its general behavior. The mechanism of dipeptide and tripeptide formation from Z-Phe and Phe-OBzl has been proposed and the supporting data demonstrated by HPLC analysis.

RESULTS AND DISCUSSION

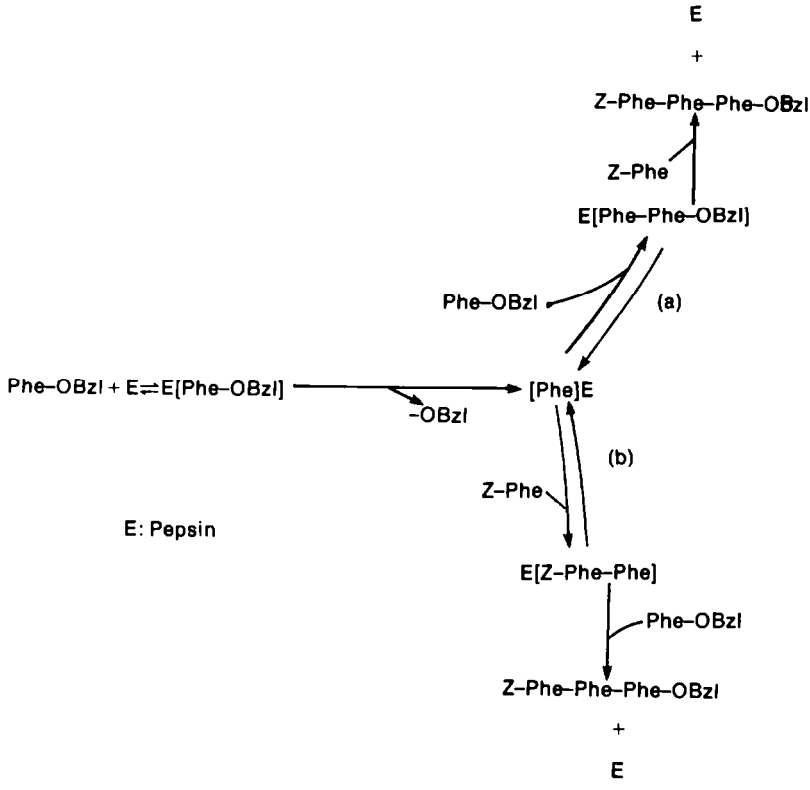
The peptides synthesized by pepsin are listed in Table 1. It was surprising that tripeptide, Z-Phe-Phe-Phe-OBzl, was obtained together with dipeptide, Z-Phe-Phe-OBzl, when Z-Phe and Phe-OBzl were used as coupling substrates. In the meanwhile, tetrapeptide, Z-Phe-Leu-Phe-Phe-OBzl, was also obtained concurrently with tripeptide, Z-Phe-Leu-Phe-OBzl, when Z-Phe-Leu and Phe-OBzl were incubated together in pepsin solution. At first, we thought that the undesired tripeptide Z-Phe-Phe-Phe-OBzl and tetrapeptide Z-Phe-Lue-Phe-Phe-OBzl were formed by impure pepsin which may be contaminated with other proteases. After a series of experiments with many grades of pepsin, the result was always the same.

In order to explain the results, two possible mechanisms are proposed for the catalytic action of pepsin and the formation of tripeptide Z-Phe-Phe-Phe-OBzl and tetrapeptide Z-Phe-Leu-Phe-Phe-OBzl (Scheme 1a, b). Since Z-Phe cannot be hydrolyzed by pepsin,[6] the formation of tripeptide Z-Phe-Phe-Phe-OBzl or tetrapeptide Z-Phe-Leu-Phe-Phe-OBzl from the transpeptidation of Z-Phe is ruled out. The ability of pepsin to hydrolyze an ester linkage for the suitable substrate has been reported by the case of the depsipeptide derivative, Z-His-Phe(NO_2)-Pla (Pla is 2-phenyl-2-lactyl), which is cleaved at the Phe (NO_2)-Pla bond.[6] The esterase activity of pepsin provided a possible explanation for the formation of tripeptide or tetrapeptide from the transpeptidation of Phe-OBzl (as shown in Scheme 1). From Scheme 1, it is expected that if the Phe-Phe-OBzl was found in the reaction solution, the pathway of formation of the tripeptide, Z-Phe-Phe-Phe-OBzl, would be Scheme 1(a). Or, if the Z-Phe-Phe was found, the pathway would be Scheme 1(b). The HPLC system

Table 1. Pepsin-catalyzed peptide synthesis

Reactants	Product (s*)	Yield
Z-Phe + Phe-OMe	Z-Phe-Phe-OMe	42%
Z-Phe + Phe-NH_2	Z-Phe-Phe-NH_2	58%
Z-Phe + Phe-OBzl	Z-Phe-Phe-OBzl	245 mg (46%)*
	Z-Phe-Phe-Phe-OBzl	154 mg (23%)*
Z-Phe + Phe + Phe-OBzl	Z-Phe-Phe-OBzl	244 mg (46%)*
	Z-Phe-Phe-Phe-OBzl	151 mg (22%)*
Z-Phe-Leu + Phe-OBzl	Z-Phe-Leu-Phe-OBzl	286 mg (44%)*
	Z-Phe-Leu-Phe-Phe-OBzl	111 mg (18%)*

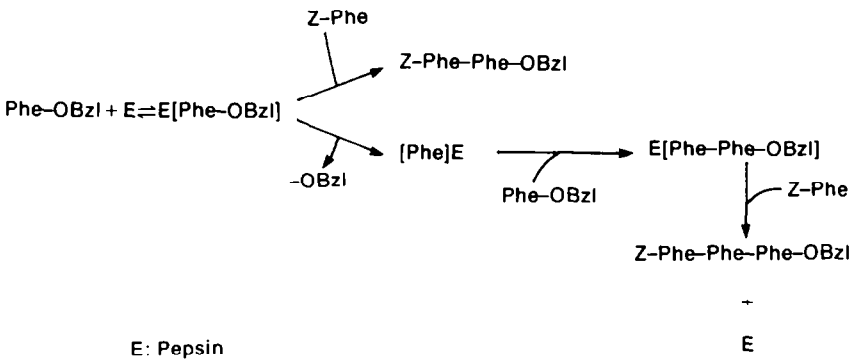
* Separated by column chromatography on silica gel.



Scheme 1.

was used as an instrument to analyze the reaction solution and the experimental conditions are briefly as follows: the reaction mixture containing 0.1 mmole Z-Phe, 0.1 mmole Phe-OBzl and 15 mg pepsin was incubated at 40°. At various reaction intervals, 500 μl aliquots were analyzed by HPLC with a Nucleosil C_{18} column. It was found that the quantities of Phe-Phe-OBzl, calculated from its peak area in HPLC analysis, were almost the same in the reaction mixture after 12, 18, 24 and 48 hr reaction periods. Owing to the appearance of Phe-Phe-OBzl and the failure to find Z-Phe-Phe which is the hydrolytic product of Z-Phe-Phe-OBzl, the tripeptide, Z-Phe-Phe-Phe-OBzl, was formed from the condensation of Phe-Phe-OBzl and Z-Phe via the pathway of Scheme 1(a). It is also reasonable to assume that tetrapeptide, Z-Phe-Leu-Phe-Phe-OBzl is formed by direct reaction of Z-Phe-Leu with Phe-Phe-OBzl.

When the equimolar of free phenylalanine was added to the solution of Z-Phe, Phe-OBzl and pepsin, the total yield ratio of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl and the peak area of Phe-Phe-OBzl did not change according to the analysis of HPLC. Apparently, free phenylalanine has no effect on the reaction. With these results, it may be proposed that when the ester bond of Phe-OBzl was cleaved by pepsin, pepsin and phenylalanine will form a stable complex which does not separate unless suitable substrates such as Phe-OBzl enter to synthesize Phe-Phe-OBzl. The addition of free



Scheme 2.

phenylalanine did not enhance the yield of Cbz-Phe-Phe-Phe-OBzl which coincided with the report of Wang and Hofmann[7] that free amino acid would not incorporate into the transpeptidation reaction, that is to say, free phenylalanine cannot enter directly into pepsin to form a complex.

The nascent Phe-Phe-OBzl, which was synthesized from pepsin-phenylalanine complex and another Phe-OBzl, will condense immediately with Z-Phe to form an insoluble product, Z-Phe-Phe-Phe-OBzl, via one subsite shift. This explains why only small amounts of Phe-Phe-OBzl were found in the reaction solution analyzed by HPLC. As described above, we propose the formation of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl in accordance with Scheme 2.

Table 2 shows the results of Z-Phe and Phe-OBzl incubated with 150, 300 and 450 mg of pepsin for 4 days under the condition described in the Experimental. The net weight of the peptides was measured by collecting the precipitates which were purified and dried, while the ratio of two peptides was calculated by HPLC according to the linear standard curve which was established with the quantity of peptides vs integrated peak area. Probably the optimal yield reached at rather low concentration of enzyme (2-2.5 mg/ml), and the higher concentration of pepsin did not enhance the total yield and effect the ratio of the two peptides.

The effect of reaction time on the pepsin-catalysed synthesis of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl is indicated in Table 3. All the reactions were carried out with equimolar amount of Z-Phe and Phe-OBzl (1 mmole, respectively) with 150 mg of pepsin. The ppts were filtered off at indicated times and dried, the yield of the two peptides being calculated by HPLC. From these data, higher yields need a longer reaction time. The ratio of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl increased to a plateau of 1.64 after 4-5 days' incubation.

The coupling between 1 mmole of Z-Phe and various concentrations of Phe-OBzl or various concentrations of Z-Phe and 1 mmole of Phe-OBzl by pepsin catalysis for 4 days was studied, as given in Table 4. The concentration of Z-Phe and Phe-OBzl had a large influence on the total yield and the ratio of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl. When the higher concentration of Phe-OBzl was used, the ratio for tripeptide increased, but the total yield was less than that of equimolar Z-Phe and Phe-OBzl. On the other hand, the higher concentration of Z-Phe not only increased the yield of dipeptide, compared with that of equimolar Z-Phe and Phe-OBzl, but also increased the total yield, to nearly a 100% level. It is now evident that the last step described in Scheme 2 to form Z-Phe-Phe-OBzl or Z-Phe-Phe-Phe-OBzl is the rate-determining step. So, the higher concentration of Z-Phe would yield a higher total. From another point of view, Z-Phe may act as a reactant and an activator in the pepsin catalyzed synthesis. It enhanced the transpeptidation of Phe-OBzl and also acted as an acceptor in peptide bond formation.[8]

We failed to obtain a product when neither amino acid is phenylalanine. The yield of pepsin-catalyzed Z-Phe-Leu-OBzl and Z-Leu-Phe-OBzl was very low (5-10%) and failed to yield a product of tyrosine or tryptophan-containing peptides (only 5-8% yield was obtained in the preparation of Z-Phe-Tyr-OBzl and Z-Tyr-Phe-OBzl). We also failed to obtain a product, when neither the amino-protected group was benzyloxycarbonyl group. When Boc-Phe, Nps-Phe and Pht-Phe was incubated with Phe-OBzl and pepsin, the formation of peptide bond failed. With the narrow scope for peptide synthesis, pepsin is not a good catalyst for the condensation of peptide bond.

Table 2. The effect of enzyme concentration on the yield of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl

Pepsin concentraction (mg/ml)	ZPhePheOBzl/ZPhePhePheOBzl (w/w)*	net yield (mg) (ZPhePheOBzl+ ZPhePhePheOBzl)
2.6	1.62	361 (62%)
5.2	1.63	370 (63%)
7.8	1.62	366 (62.5%)

*Calculated by HPLC method

Table 3. Effect of reaction time on the pepsin-catalyzed synthesis of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl

Reaction time (day)	ZPhePheOBzl/ZPhePhePheOBzl (w/w)*	net yield (mg) (ZPhePheOBzl + ZPhePhePheOBzl)
1/8	0.57	86 (14%)
1/4	0.75	106 (15%)
1/2	1.12	133 (22%)
3/4	1.40	161 (27%)
1	1.48	224 (38%)
2	1.54	276 (47%)
3	1.60	315 (54%)
4	1.62	320 (54.5%)
5	1.64	332 (55%)
6	1.64	348 (59.5%)
7	1.61	360 (61.5%)
14	1.61+	399 (69%)

*Calculated by HPLC method.

+Separated by column chromatography on silica gel.

Fruton[6] suggested that more research incorporating new approaches to the resolution of the mechanism of pepsin is an absolute requirement. The experiment on the pepsin-catalyzed peptide bond formation and the action of pepsin toward ester substrate show that Fruton's remarks have validity.

EXPERIMENTAL

All mps were measured with a Bucki m.p. apparatus and were uncorrected. Amino acid analysis was carried out with a semi-automatic amino acid analyzer, Yanagimoto Model LC5A according to the accelerated method.[9] Hydrolysis was performed with constant boiling HCl at 110° for 12 hr in an evacuated sealed tube. Optical rotation was measured by JASCO DIP 180 automatic digital polarimeter at 589 nm.

TLC was performed on silica gel GF_{254} (E. Merck) coated on a glass plate and was developed with the following solvent system: (1) $CHCl_3$–MeOH (9:1, v/v); (2) $CHCl_3$–EtOAc (7:3, v/v); (3) CH_2Cl_2–*n*-hexane–EtOAc (5:3:2, v/v/v); (4) *n*-BuOH–AcOH–water (4:1:1, v/v/v); (5) *n*-BuOH–pyridine–AcOH–water (30:20:6:24, v/v/v/v). Ninhydrin reagent (0.1% in EtOH) was used to locate the carboxyl-protected amino acid and free amino acid. Amino-protected amino acid or peptide was located by chlorine-tollidine method.[10]

A Waters Assoc. (Milford, MA, U.S.A.) HPLC System was used for the analytical separations, which consisted of two M6000A solvent delivery units, an M600 solvent programmer and a U6K Universal liquid chromatograph injector, coupled to an M450 variable-wavelength UV spectrophotometer and an Omniscribe two-channel chart recorder (Houston Instruments, Austin, TX, U.S.A.). Integration was performed electronically

Table 4. Effect of substrate concentration on the pepsin-catalyzed synthesis of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl

Substrate concentration (mmole)		ZPhePheOBzl/ ZPhePhePheOBzl (w/w)*	net yield (mg) (ZPhePheOBzl + ZPhePhePheOBzl)
ZPhe	PheOBzl		
1/5	1	0.33	24 (19%)+
1/3	1	0.76	114 (51%)+
1/2	1	1.08	161 (55%)+
1	1	1.66	378 (65%)
1	1/2	1.95	230 (78%)++
1	1/3	2.40	165 (89%)++
1	1/5	3.00	108 (98%)++

*Calculated by HPLC method.

+The percentage was calculated based on the molarity of Z-Phe.

++The percentage was calculated based on the molarity of Phe-OBzl.

Table 5. The physical constants of the peptides synthesized by pepsin

Peptides	m.p.	R_f* (tlc)	Optical rotation $[\alpha]_D^{25}$	Amino acid analysis
Z-Phe-Phe-OMe	150-151	0.74(2) 0.59(3)	-17.4 (C1, DMF)	
Z-Phe-Phe-NH_2	223.5-225	0.70(1) 0.10(2)	-11.5 (C1, ACOH)	
Z-Phe-Phe-OBzl	162-162.5	0.83(2) 0.60(3)	-18.1 (C2, DMF)	
Z-Phe-Phe-Phe-OBzl	187-188	0.71(2) 0.21(3)	-21.6 (C2, DMF)	
Z-Phe-Leu-Phe-OBzl	165-166	0.72(2) 0.22(3)	-19.0 (C1, DMF)	$Phe_{2.00}$ $Leu_{1.00}$
Z-Phe-Leu-Phe-Phe-OBzl	202.5-204	0.58(2) 0.12(3)	-18.4 (C1, DMF)	$Phe_{3.00}$ $Leu_{1.00}$

* (1): tlc developed with solvent 1.

(2): tlc developed with solvent 2.

(3): tlc developed with solvent 3.

with an SP4100 Computing Integrator. (Santa Clarn, CA, U.S.A.). Sample injection was made using a Microliter 802 Syringe (Hamiltin, Reno, NV, U.S.A.). The Nucleosil 10 C_{18} column (25 cm × 4 mm) was purchased from Macherey-Nagel GMBH & Co. KG, Germany. The silica gel column (25 cm × 4 mm) was obtained from E. Merck, Germany.

Pepsin (2 times crystallyzed) was purchased from Sigma, U.S.A. and its potency was described as 3180 Units/mg Protein. Phenylalanine (Phe) and leucine (Leu) were obtained from Kyowa Fermentation Company, Tokyo, Japan. Trifluoroacetic acid (TFA) was from Sigma, U.S.A. N,N'-dicyclohexylcarbodimide (DCCI) and N-hydroxylsuccinimide (HONSu) were supplied by Pierce Chemical Company, U.S.A. Citric acid monohydrate and sodium citrate were from Wako Pure Chemical Industries, Japan. Benzyloxycarbonyl chloride (Z-Cl),[11] *o*-nitrophenylsulfenyl chloride (Nps-Cl),[12] and 2,4,6-trimethylbenzyl chloride (TMB-Cl)[13] were synthesized using established procedures.

Preparation of amino acid derivatives. Z-Phe was prepared from Z-Cl and phenylalanine according to the method cited.[14] The 2,4,6-trimethylbenzyl ester derivative of lecucine (Leu-OTMB) was prepared according to the method cited.[15] The benzyl ester derivative of phenylalanine (Phe-OBzl) was prepared following the method described.[16] The benzyl ester was prepared as tosylate and converted into hydrochloride salt. The methyl ester was prepared by thionyl chloride method.[17]

Preparation of Phe-NH_2 *hydrochloride.* Nps-Phe-OSu was prepared from Nps-Phe and NONSu by DCCI method.[18] After removal of the precipitated dicyclohexylurea by filtration, the filtrate was evaporated, dissolved the oily Nps-Phe-OSu in THF, and the mixture was saturated at −5° with dry ammonia for 2 hr, then the ammonia saturated soln was kept in a refrigerator overnight. The insoluble solid was removed by filtration, the filtrate was evaporated. The yellow oil was treated with a MeOH saturated with HCl at room temp. Upon addition of ether, the product precipitated out almost instantly. After allowing the mixture to stand in a refrigerator overnight, it was filtered and the product was purified by recrystallization from MeOH/ether, yield 86%, m.p. 238–240°, R_f 0.52 (solvent 4).

Preparation of Z-Phe-Leu. Z-Phe-Leu-OTMB was prepared by papain catalysis as described.[19] A mixture of Z-Phe-Leu OTMB (505 mg), CH_2Cl_2 (1.5 ml), and TFA (1.5 ml) was stirred at room temp for 20 min. The reagent was removed *in vacuo*, dry ether (50 ml) was added, then the crude product was filtered, washed with ether and recrystallized from EtOAc/*n*-hexane, yield 350 mg (85%), m.p. 142–143°, $[\alpha]_D^{25}$ −19.0 (*C*2, MeOH), R_f 0.71 (solvent 5).

Enzymic reaction. 1N-NaOH (3 ml) was added to 1 mmole of Z-X (X is Phe or Phe-Leu) to dissolved the reactant, then 20 ml water, 30 ml 0.1 M citrate buffer (pH 4.0), 1 mmole Phe-OY hydrochloride (Y is Bzl or Me) or Phe-NH_2 hydrochloride, and 5 ml MeOH were added. Finally pepsin (150 mg) was added to the mixture. The soln was shaken occasionally by hand for several days at 40°. The ppt was collected every other day. Finally, the soln was discarded when no more ppt was formed after 3–4 days. The combined ppts were washed in succession with 1N $NaHCO_3$, water, 1N H_2SO_4, and water. Z-Phe-Phe-OMe and Z-Phe-Phe-NH_2 were purified by recrystallization. For the reactions of Z-Phe or Z-Phe-Leu with Phe-OBzl, there were 2 spots on TLC, respectively. The R_f values of the products obtained from the reaction of Z-Phe and Phe-OBzl corresponded to those of the chemical synthesized Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl. For the reaction of Z-Phe-Leu and Phe-OBzl, the R_f values of the 2 spots corresponded to the synthetic Z-Phe-Leu-Phe-OBzl and Z-Phe-Leu-Phe-Phe-OBzl. The products were separated and purified by column chromatography on silica gel using $CHCl_3$-*n*-hexane-EtOAc (11:8:1, v/v/v) as an eluent.

Quantitative determination of coupling yield by HPLC *method.* Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl could be separated by HPLC on silica gel column using $CHCl_3$-*n*-hexane (70:30, v/v) as an eluent. The wavelength of the detector was set at 254 nm. The flow rate was 2.0 ml/min, the retention time of Z-Phe-Phe-OBzl was 2.29 min and that of Z-Phe-Phe-Phe-OBzl was 4.78 min. The instrument was calibrated by introducing samples of chemical synthetic Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl at known concentration and the integrated peak areas on the computing integrator traces obtained. The resultant linear plot of area vs concentration defined the unknown concentration of the substance in samples of the pepsin-catalysed reactions. The enzymic mixtures were filtered at indicated times and the dry products analyzed by HPLC.

Kinetic study of action of pepsin. The samples of a mixture containing 0.3 ml 1N NaOH, 2 ml water, 3 ml citrate buffer (pH 4.0), 0.5 ml MeOH, 0.1 mmole Z-Phe and Phe-OBzl and 15 mg pepsin, were removed at 0, 18, 24 and 48 hr. The collected ppts were analyzed by HPLC (as described) to determine the total yield and the ratio of Z-Phe-Phe-OBzl and Z-Phe-Phe-Phe-OBzl. The soluble part was filtered through a millipore filter paper (HA type) and analyzed by HPLC with a Nucleosil C_{18} column. The eluent, 55% (v/v) MeOH and 45% 0.1M potassium phosphate (pH 6.0), revealed Phe-Phe-OBzl and another eluent, 50% (v/v) MeOH and 50% (v/v) 0.1M ammonium acetate (pH 6.8) revealed Z-Phe-Phe.

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