J. Biochem. **84**, 1277–1283 (1978)

Peptide Bond Synthesis Catalyzed by α-Chymotrypsin

Tatsushi OKA and Kazuyuki MORIHARA

Shionogi Research Laboratory, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka, Osaka 553

Received for publication, June 22, 1978

α-Chymotrypsin [EC 3.4.21.1] catalyzed the syntheses of peptide bonds with various *N*-acylated amino acids or peptides having aromatic or hydrophobic amino acid residues at the C-terminal position as carboxyl components, and amino acid derivatives, peptides or their derivatives as amine components. A neutral pH was most efficient and quite high concentrations of α-chymotrypsin and starting materials were required for synthesis. For amine components, hydrophobic or bulky amino acid residues were useful at the N-terminal position. Stereospecificity was also observed at the N-terminal position of amine components. Peptide synthesis was not usually seen when the products were soluble in the reaction mixture. This could be partly overcome by increasing the concentration of either the carboxyl or the amine component to more than ten times that of the other.

Proteases are well-known as enzymes which can split peptide bonds in proteins or polypeptides. The reverse reaction, *i.e.*, peptide bond synthesis, can also be catalyzed by the same enzyme, as was first demonstrated by Bergmann and Fraenkel-Conrat (*1*) in 1937 with papain [EC 3.4.22.2]-catalyzed amide (anilide) bond synthesis. Since then, many reports have appeared on the reverse reaction of proteases, for instance, in the asymmetric synthesis of a series of anilides (*2–4*), transamidation (*5*), transpeptidation (*6–11*), synthesis of peptides (*12–20*), polymerization of dipeptides (*21*) or plastein formation (*22–24*).

As regards peptide synthesis by chymotrypsin [EC 3.4.21.1], in 1938 Bergmann and Fruton (*12*) synthesized Bz-Tyr-Gly-NHC_6H_5 from Bz-Tyr-OH and Gly-NHC_6H_5, and a kinetic study was done in 1961 by Gawron *et al.* (*25*). Recently, Saltman *et al.* (*17*, *18*) have shown that α-chymotrypsin catalyzes the coupling step of the following scheme.

P-Ar-OH + H-Gly-Ar-NH_2 ⟶ P-Ar-Gly-Ar-NH_2

Cbz-Ar-OH + H-Gly-OCH_3 (or H-Ala-OCH_3) ⟶ Cbz-Ar-Gly-OCH_3 (or Cbz-Ar-Ala-OCH_3)

P, α-amino blocking group; Ar, aromatic amino acid residue.

In a previous paper (*19*), we showed that α-chymotrypsin is a potent catalyst for peptide synthesis using *N*-acylated amino acid or peptide ester substrates (the carboxyl terminal position of peptide substrates being occupied by an aromatic or hydrophobic amino acid residue) as carboxyl components and amino acid derivatives or peptides or their derivatives as amine components. The present study deals with the synthesis of peptides by α-chymotrypsin using *N*-acylated amino acids or peptides as carboxyl components for comparison with the above ester method.

MATERIALS AND METHODS

Enzyme—α-Chymotrypsin (recrystallized three times; Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was used without further purification.

Amino Acid Derivatives, Peptides, or Their Derivatives—Cbz-Phe-OH, Cbz-Tyr-OH, Cbz-Gly-Phe-OH, Cbz-Gly-Pro-Leu-OH, Leu-NH_2-HCl, Leu-Gly, Leu-Gly-Gly, Leu-OEt, and L-leucine were supplied by the Protein Research Foundation, Minoh, Osaka. Val-OBu^t-HCl (*19*), Ala-NH_2-HCl (*26*), Val-NH_2-HCl (*26*), Ile-NH_2-HCl (*26*), Gly-NH_2-HCl (*19*), DL-Leu-NH_2-HCl (*19*), D-Leu-NH_2-HCl (*19*), Pro-NH_2-HCl (*19*), and Gly-Leu-NH_2-HCl (*19*) were prepared as described previously.

Enzymatic Synthesis of Peptides—The reaction mixture (1 ml) contained 0.1 M carboxyl component, 0.1 M amine component, 0.2 mM α-chymotrypsin, and 0.2 M Tris-maleate buffer, pH 7. The reaction mixture was kept at 37°C for 20 h. To stop the reaction, 0.5 ml of 1 M HCl and 2 ml of ethanol were added. If necessary, more ethanol was added to dissolve the precipitate (products) completely. Except where otherwise specified, the product yield was determined by the TLC method, as follows. The carboxyl component and its product in the reaction mixture were separated by TLC in various solvents: a mixture of chloroform and acetic acid (I, 3 : 1; II, 4 : 1; III, 6 : 1; IV, 9 : 1; V, 19 : 1; all v/v), and a mixture of chloroform, methanol, and acetic acid (VI, 80 : 20 : 3; VII, 90 : 10 : 3). After spraying with conc. H_2SO_4, the plate was heated at 200°C for 10–30 min. The color intensities of the spots corresponding to the starting carboxyl component and its product were determined using a dual-wavelength TLC scanner (A_{465}–A_{450}), model CS-900, manufactured by Shimadzu Co, Kyoto. The product yield was determined by calculating the ratio against the total intensities of the spots of *N*-acylated materials.

The yield of Cbz-Phe-Leu-NH_2 was also determined using an amino acid analyzer (Japan Electron Optics Laboratory, model JLC-3). After catalytic hydrogenolysis of the reaction mixture in the presence of Pd-black, the mixture of phenylalanine, Leu-NH_2 and Phe-Leu-NH_2 was separated on a column (0.8 × 15 cm) of chromosorv #2611 resin using citrate buffer (Na concentration, 0.93 N) containing 10% ethyl alcohol, pH 6.3, at 55°C. The flow rate was 70 ml/h. The elution time of phenylalanine, Leu-NH_2 and Phe-Leu-NH_2 was 23, 71, and 144 min, respectively.

Identification—After the reaction mixture had been concentrated *in vacuo*, the residue was dissolved in ethyl acetate, then successively washed with 1 M HCl, water, 5% (w/v) $NaHCO_3$, and water, and dried on anhydrous Na_2SO_4. The product was crystallized from suitable solvents after concentration of the ethyl acetate portion. The purity was checked by TLC and identification was carried out in the usual manner. The R_f values of products were calculated by using the carboxyl components as controls ($R_f = 1.0$); roman numerals show the solvent used for TLC as mentioned above.

Solubility Test—An excess amount of the products was suspended in 50 ml of distilled water in a 200 ml Erlenmeyer flask, and the suspension was shaken for 20 h at 37°C. After discarding the residue by filtration, a definite volume of the clear supernatant was concentrated *in vacuo*, and hydrolyzed in 6 N HCl at 110°C for 20 h. The amino acid composition was quantitatively determined using an amino acid analyzer.

RESULTS

Optimal Conditions for Peptide Synthesis—The synthesis of Cbz-Phe-Leu-NH_2 from Cbz-Phe-OH and an equimolar amount of Leu-NH_2 was studied by TLC under various experimental conditions. The low solubility of the product (6.5×10^{-6} M) caused it to precipitate in the reaction mixture. The results indicate that a neutral pH range is suitable for synthesis (Fig. 1) and a long reaction time (more than ten hours) is required for its completion (Fig. 2), where the rate of synthesis depends on the enzyme concentration. Figure 3 and Table I indicate that the concentration of the starting materials should be higher than 0.05 M. Figure 3 further shows that more enzyme is required with an increase in the concentration of the starting materials; optimal enzyme concentrations are 0.05, 0.2, and 0.6 mM in reaction mixtures containing 0.05, 0.1, and 0.2 M starting materials, respectively. In the case of 0.3 or 0.4 M starting materials, an enzyme concentration of more

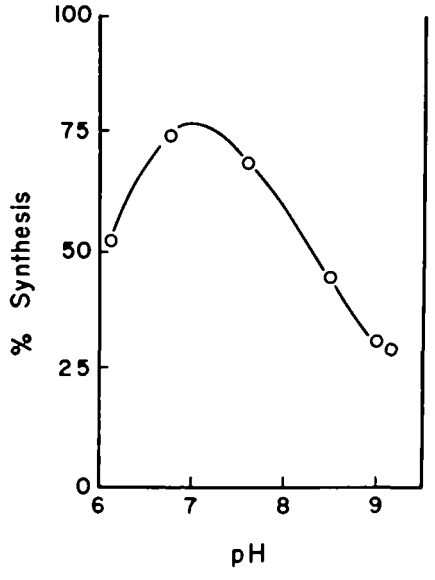

Fig. 1. Effect of pH on peptide synthesis. The reaction mixture (1 ml) contained 0.1 M Cbz-Phe-OH, 0.1 M Leu-NH_2, 0.4 mM α-chymotrypsin, and 0.2 M Tris-buffer at various pH values. The pH was determined with a glass electrode. The reaction mixture was kept at 37°C for 20 h.

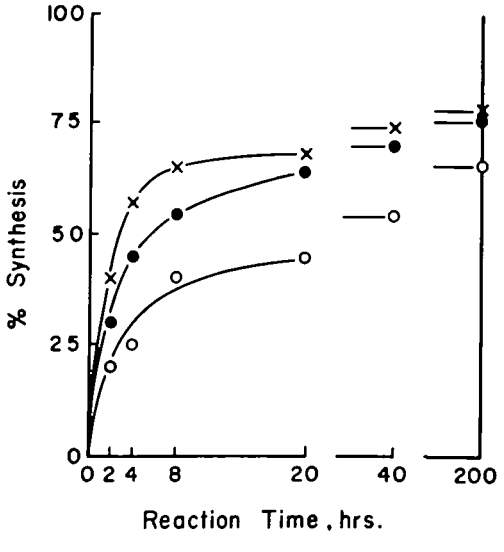

Fig. 2. Effect of reaction time on peptide synthesis. The reaction mixture (1 ml) contained 0.1 M Cbz-Phe-OH, 0.1 M Leu-NH_2, and 0.2 M Tris-buffer, pH 7, and was kept at 37°C. The enzyme concentrations were 0.1 mM (○), 0.2 mM (●), and 0.4 mM (×).

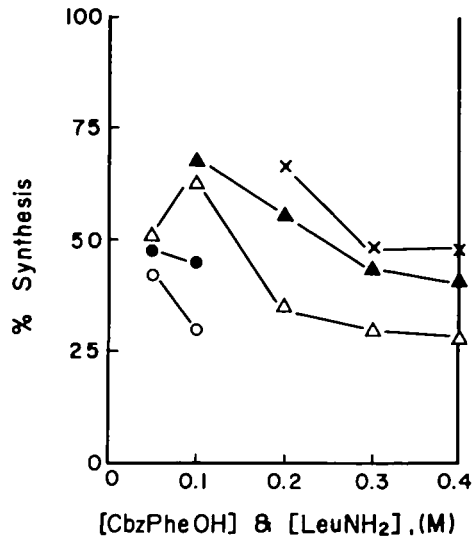

Fig. 3. Effect of the concentration of starting materials on peptide synthesis. The reaction mixture (1 ml) contained 0.2 M Tris-buffer, pH 7, and various concentrations of starting materials. The enzyme concentrations were 0.05 mM (○), 0.1 mM (●), 0.2 mM (△), 0.4 mM (▲), and 0.6 mM (×). The reaction was continued for 20 h at 37°C.

than 0.6 mM may be necessary to obtain maximum synthesis.

The product yield was small with less than 10 mM starting materials. This was partly overcome by increasing the concentration of either component, as shown in Table I; the experiment was performed using an amino acid analyzer to determine the yield more precisely than by TLC. A considerable yield was thus obtained even in a reaction mixture containing 10 mM of either component when the concentration of the other component was 0.1 M; in this case the product precipitated in the reaction mixture.

Effect of the Kind of Amine Components—The following experiment was undertaken to investigate the effect of the amine component on the synthesis using Cbz-Phe-OH as the carboxyl component. Table II indicates that a considerable yield was obtained when Val-NH_2, Ile-NH_2, and Gly-Leu-NH_2 were used as the amine component, as seen with Leu-NH_2, with the products precipitating in the reaction mixture. The results of identification are summarized in Table III. When DL-Leu-NH_2 was used, a considerable yield of the corresponding

TABLE I. Effect of the concentration of either component on the synthesis of Cbz-Phe-Leu-NH_2. The reaction mixture (1 ml) contained 0.2 mM α-chymotrypsin and 0.2 M Tris-buffer, pH 7, and was kept at 37°C for 20 h. The concentrations of starting materials are given in the table. The product yield was determined using an amino acid analyzer.

[Cbz-Phe-OH] (mM)	[Leu-NH_2] (mM)	Yield (%)
100	100	88
50	50	72
25	25	49
10	10	18
5	5	8
100	50	88
100	25	85
100	10	56
100	5	14
100	2	7
25	100	72
10	100	45
5	100	14

TABLE II. Effect of the kind of amine components on peptide synthesis. The reaction mixture (1 ml) contained 0.1 M Cbz-Phe-OH, 0.1 M amine component, 0.2 mM α-chymotrypsin, and 0.2 M Tris-buffer, pH 7, and was kept at 37°C for 20 h.

Amine component	Synthesis (%)
L-Leucine	0
Leu-OEt	<10
Val-OBu^t	10
Leu-NH_2	71
Gly-NH_2	<10
Ala-NH_2	12
Val-NH_2	70
Ile-NH_2	75
Pro-NH_2	0
DL-Leu-NH_2	76[a]
D-Leu-NH_2	30[b]
Leu-Gly	14
Gly-Leu-NH_2	78

[a] The reaction mixture contained 0.1 M Cbz-Phe-OH and 0.22 M DL-Leu-NH_2. The product was L-form, as shown in Table III. [b] The product was D-form, as shown in Table III.

TABLE III. Identification of products.

Product	R_f	m.p. (°C)		Elementary analysis		
Cbz-Phe-Ala-NH_2	0.60 (IV)	211-213	Calc.	65.03	6.28	11.37
			Found	65.20	6.32	11.19
Cbz-Phe-Val-NH_2	0.68 (II)	243-245	Calc.	66.48	6.85	10.57
			Found	66.18	6.81	10.66
Cbz-Phe-Ile-NH_2	0.78 (II)	226.5-227.5	Calc.	67.13	7.11	10.21
			Found	66.87	7.06	10.38
Cbz-Phe-Leu-NH_2[a]	0.68 (II)	192.5-193.5	Calc.	67.13	7.11	10.21
			Found	67.09	6.83	10.23
Cbz-Phe-Leu-NH_2[b]	0.68 (II)	194.5-196	Calc.	67.13	7.11	10.21
			Found	67.33	6.85	10 30
Cbz-Phe-D-Leu-NH_2[c]	0.79 (IV)	177-179	Calc.	67.13	7.11	10 21
			Found	66.89	7.02	10.29
Cbz-Phe-Gly-Leu-NH_2	0.33 (III)	212-214	Calc.	64.09	6.88	11.96
			Found	63.52	6.61	11.74
Cbz-Gly-Phe-Leu-NH_2	0.85 (VI)	211-213	Calc.	64.09	6.88	11.96
			Found	64.38	6 88	11.59
Cbz-Gly-Pro-Leu-Leu-NH_2	0.69 (II)	199-201	Calc.	61.00	7.77	13.17
			Found	59.98	7.76	13.33

[a] Prepared with L-Leu-NH_2 as the amine component. $[\alpha]_D = -21.0 \pm 0.6°$ (1.007% in methanol). [b] Prepared with DL-Leu-NH_2 as the amine component. $[\alpha]_D = -21.6 \pm 0.6°$ (1.048% in methanol). [c] Prepared with D-Leu-NH_2 as the amine component. $[\alpha]_D = +46.0 \pm 1.1°$ (0.784% in methanol).

L-form was obtained as a precipitate, as shown in Tables II and III. Nonetheless, Cbz-Phe-D-Leu-NH_2 was also synthesized when D-Leu-NH_2 was used, although the yield was smaller than that of the corresponding L-form (Tables II and III).

The product yield was negligible when L-leucine, Leu-OEt, Val-OBu^t, Gly-NH_2, Ala-NH_2, Pro-NH_2 or Leu-Gly was used (Table II). Leu-OEt might have been hydrolyzed to L-leucine by the enzyme before being used as an amine component. It is well-known that ester substrates are extremely sensitive to α-chymotrypsin in comparison with amide or peptide substrates. The solubilities of Cbz-Phe-Gly-NH_2, Cbz-Phe-Ala-NH_2, and Cbz-Phe-Leu-Gly were 1.1×10^{-3}, 7.1×10^{-5}, and 4×10^{-3} M, respectively. Negligible synthesis of these products might be partly due to their high solubilities in the reaction mixture.

TABLE IV. Effect of the concentration of amine components on peptide synthesis. The concentrations of amine components are shown in the table. The other experimental conditions were as described in Table II.

Concentration of amine components (M)	Synthesis (%)	
	Ala-NH_2	Leu-Gly[a]
0.1	6	14
0.2	39	18
0.4	46	27
1.2		52
1.8	85	

[a] R_f, 0.4 (IV). Amino acid analysis of the hydrolysate (6 M-HCl, 120°C, 20 h) confirmed its composition.

TABLE V. Effect of the kind of carboxyl components on peptide synthesis. The experimental conditions were as described in Table II.

Carboxyl component	Amine component	Synthesis (%)
Cbz-Tyr-OH	Gly-Leu-NH_2	55[a]
Cbz-Gly-Phe-OH	Leu-NH_2	65[b]
Cbz-Gly-Pro-Leu-OH	Leu-NH_2	78

[a] R_f, 0.58 (VII). [b] The reaction mixture was scaled up to 10 ml. The precipitate was washed with 5% NH_3, 1 M HCl, and water, successively. After drying, the product was weighed to determine the yield directly.

Next, the effect of the concentration of the poor amine components was examined using 0.1 M Cbz-Phe-OH as the carboxyl component. The results, summarized in Table IV, indicate that a considerable yield was obtained when more than 1 M Ala-NH_2 or Leu-Gly was used as the amine component, but not with L-leucine, Leu-OEt, Gly-NH_2 or Pro-NH_2. Cbz-Phe-Ala-NH_2 precipitated in the reaction mixture, while Cbz-Phe-Leu-Gly dissolved in it.

Effect of the Kind of Carboxyl Components—Cbz-Tyr-OH, Cbz-Gly-Phe-OH, and Cbz-Gly-Pro-Leu-OH were used as the carboxyl component as shown in Table V. Identification data are also given in Table III. They were all useful, as in the case of Cbz-Phe-OH. The synthesis of Cbz-Gly-Phe-Leu-NH_2 was carried out on a 10-ml scale to determine the yield directly.

DISCUSSION

In previous papers (*19*, *20*), we showed that *N*-acylated ester substrates are useful as carboxyl components for peptide synthesis by either α-chymotrypsin or trypsin. The optimal results were compared with those studied in this work, as shown in Table VI. The comparison indicates that a more alkaline pH is suitable and the reaction is very fast with ester substrates, while a neutral pH is useful and the reaction is slow in the synthesis of this study. More interestingly, ten or twenty times the amount of enzyme is required for the present synthesis compared with ester substrates. However, a high concentration of starting materials is useful in both cases.

TABLE VI. Conditions for peptide synthesis.[a]

Conditions	Carboxyl-component	
	Ac-Phe-OEt (*19*) Bz-Arg-OEt (*20*) (Chymotrypsin / Trypsin)	Cbz-Phe-OH (Chymotrypsin)
Opt. pH	*ca.* 10	*ca.* 7
[E]	0.01–0.02 mM	0.2–0.4 mM
Reaction time	2–5 min	>10 h
Concentration	>0.1 M	>0.1 M

[a] Leu-NH_2 was used as the amine component.

A similar result has been obtained by Saltman *et al.* (*17*) in the α-chymotrypsin-catalyzed synthesis of P-Ar-Gly-Ar-NH_2 as mentioned in the introduction to this paper. They stressed that the coupling reaction occurred under a much more restricted set of conditions than the hydrolytic reaction, *e.g.*, only within a small pH range (*ca.* 6.5–7.5) and with particular buffering agents such as carbonate buffer. However, the present study indicates that Tris-buffer is also useful in the coupling reaction. Isowa *et al.* (*14–16*) have further shown that various proteases such as papain, nagarse, thermolysin, pepsin, *etc.* can be used in various types of coupling reaction. The optimal conditions for synthesis, except in the case of pepsin, were very similar to those determined in the present study using α-chymotrypsin. However, Saltman *et al.* (*17*, *18*) and Isowa *et al.* (*14–16*) did not give any information on the mechanism of the synthetic reaction. On the other hand, Gawron *et al.* (*25*) carried out a kinetic study on the condensation of Bz-Tyr-Gly-NHC_6H_5 from Bz-Tyr-OH and Gly-NHC_6H_5, and proposed that the synthetic process is the reverse of that of α-chymotrypsin-catalyzed hydrolysis.

The mechanism of synthesis may be interpreted in terms of the following scheme, where the acylation process has been proposed (*27*) on the basis of incorporation of ^{18}O from water into the carboxyl group of *N*-acylated amino acids in the presence of chymotrypsin.

$$\text{Cbz-Phe-OH} + \text{E} \underset{}{\overset{K_s}{\rightleftharpoons}} \text{Cbz-Phe-OH}\cdot\text{E} \overset{k_2}{\rightleftharpoons}$$

$$\text{Cbz-Phe-E} \begin{cases} \xrightarrow{k_3[H_2O]} \text{Cbz-Phe-OH} \\ \xrightarrow{k_4[X]} \text{Cbz-Phe-X} \end{cases}$$

It has been shown (*28*) that the rate-determining step is acylation (k_2) in amide or peptide bond hydrolysis. Since Cbz-Phe-OH possesses a terminal charged group and is thus regarded as a virtual substrate, the k_2 value may be much smaller than those of amide or peptide substrates. Thus, Cbz-Phe-E (acyl-enzyme) may not accumulate in the reaction mixture, but may be immediately consumed for the deacylation process. Preferential synthesis may occur rather than hydrolysis, depending upon the nature of X ($k_4[X] > k_3[H_2O]$); this was discussed in the previous paper (*19*). In the whole reaction, the rate of synthesis must be determined by the slow step k_2. This may be why the synthesis takes much longer than that using ester substrates.

To achieve successful synthesis, the rate of the synthetic reaction must be faster than that of secondary hydrolysis of the products. When the products are soluble in the reaction mixture, the rate of secondary hydrolysis may be markedly faster than that of synthesis. For example, papain has been found (*29*) to be over 1,000 times more efficient in hydrolyzing Bz-Arg-NH_2 than in synthesizing hippuryl anilide. The rate of synthesis becomes faster than that of secondary hydrolysis when the solubility of the products is very small due to a lack of substrates in the soluble state. Thus, the synthesis of Cbz-Phe-Leu-NH_2 is considerable when the concentration of the starting materials is above 25 mM, but is negligible when the concentration is below 10 mM. Therefore, the rate of synthesis may not exceed that of the secondary hydrolysis at 0.01 mM (solubility of Cbz-Phe-Leu-NH_2) until the concentration of the starting materials is increased above 25 mM.

In the deacylation process, the k_4 values of amine components are usually related to their P_1'-specificity (notation of Schechter and Berger, *30*); this has also been observed in synthesis using ester substrates (*19*). Hydrophobic or bulky amino acid residues are efficient and stereospecificity can be observed at this position. However, D-Leu-NH_2 can also be used as an amine component, although the rate of synthesis is so slow that dissolution of DL-Leu-NH_2 can be seen. Even with inadequate amine components, *e.g.* Ala-NH_2, synthesis occurred when their concentration was considerably increased over that of the corresponding carboxyl component. This was not observed with L-leucine, Leu-OEt, Gly-NH_2 or Pro-NH_2 as the amine component.

When ester substrates are used as carboxyl components, enzymatic synthesis is possible even though the products are soluble in the reaction mixture, since the rate of synthesis is much faster than that of the secondary hydrolysis. This is not usually the case in synthesis with C-terminal free carboxyl components, as in this study. However, this problem was overcome by increasing the concentration of amine components. For example, Cbz-Phe-Leu-Gly (solubility, 4×10^{-2} M)

was synthesized in considerable yield in the soluble state from Cbz-Phe-OH (0.1 M) and Leu-Gly (1.2 M). A high concentration of amine components would not only increase the rate of the deacylation process but would also inhibit the secondary hydrolysis of the products. Immobilized chymotrypsin was found (*31*) to catalyze the esterification of Ac-Tyr-OH in a medium containing a high concentration of alcohols, which may be useful for the synthesis of peptides in the soluble state.

The present study indicates that enzymatic synthesis of peptides is possible using C-terminal free carboxyl components, although the conditions are considerably different from those required for ester substrates as carboxyl components.

REFERENCES

1. Bergmann, M. & Fraenkel-Conrat, H. (1937) *J. Biol. Chem.* **124**, 321–329
2. Kimmel, J.R. & Smith, E.L. (1957) *Adv. Enzymol.* **19**, 308–312
3. Murachi, T. (1958) *Arch. Biochem. Biophys.* **78**, 115–124
4. Kuno, S. & Murachi, T. (1961) *Arch. Biochem. Biophys.* **94**, 201–206
5. Johnston, R.B., Mycek, M.J., & Fruton, J.S. (1950) *J. Biol. Chem.* **185**, 629–641
6. Johnston, R.B., Mycek, M.J., & Fruton, J.S. (1950) *J. Biol. Chem.* **187**, 205–211
7. Fruton, J.S., Johnston, R.B., & Fried, M. (1951) *J. Biol. Chem.* **190**, 39–53
8. Waley, S.G. & Watson, J. (1954) *Biochem. J.* **57**, 529–538
9. Blau, K. & Waley, S.G. (1954) *Biochem. J.* **57**, 538–541
10. Neumann, H., Levin, Y., Berger, A., & Katchalski, E. (1959) *Biochem. J.* **73**, 33–41
11. Fruton, J.S., Fujii, S., & Knappenberger, M.H. (1961) *Proc. Natl. Acad. Sci. U.S.* **47**, 759–761
12. Bergmann, M. & Fruton, J.S. (1938) *J. Biol. Chem.* **124**, 321–329
13. Behrens, O.K. & Bergmann, M. (1939) *J. Biol. Chem.* **129**, 587–602
14. Isowa, Y., Ohmori, M., Ichikawa, T., Kurita, H., Sato, M , & Mori, K. (1977) *Bull. Chem. Soc. Jpn.* **50**, 2762–2765
15. Isowa, Y., Ohmori, M., Sato, M., & Mori, K. (1977) *Bull. Chem Soc. Jpn.* **50**, 2766–2772
16. Isowa, Y., Ichikawa, T., & Ohmori, M. (1978) *Bull. Chem. Soc. Jpn* **51**, 271–276
17. Saltman, R., Vlach, D., & Luisi, P.L. (1977) *Biopolymers* **16**, 631–638
18. Luigi, P.L., Saltman, R., Vlach, D., & Guarnaccia, R. (1977) *J. Mol. Catalysis* **2**, 133–138
19. Morihara, K. & Oka, T. (1977) *Biochem. J.* **163**, 531–542
20. Oka, T. & Morihara, K. (1977) *J. Biochem.* **82**, 1055–1062
21. Heinrich, C.P. & Fruton, J.S. (1968) *Biochemistry* **7**, 3556–3565
22. Horowitz, J. & Haurowitz, F. (1959) *Biochim. Biophys. Acta* **33**, 231–237
23. Fujimaki, M , Yamashita, M , Arai, S., & Kato, H. (1970) *Agric. Biol. Chem.* **34**, 1325–1332
24. Yamashita, M., Arai, S., Tanimoto, S., & Fujimaki, M. (1974) *Biochim. Biophys. Acta* **358**, 105–112
25. Gawron, O., Glaid, A.J., III, Boyle, R.E., & Odstrchel, G. (1961) *Arch. Biochem. Biophys.* **95**, 203–210
26. Morihara, K., Oka, T., & Tsuzuki, H. (1960) *Arch. Biochem. Biophys.* **135**, 311–323
27. Bender, M.L. & Kemp, K.C. (1957) *J. Am. Chem. Soc.* **79**, 116–120
28. Zerner, B., Richard, P.M.B., & Bender, M.L. (1964) *J. Am. Chem. Soc.* **86**, 3674–3679
29. Carty, R.P. & Kirschenbaum, D.M. (1964) *Biochim. Biophys. Acta* **85**, 446–461
30. Schechter, I. & Berger, A. (1967) *Biochem. Biophys. Res. Commun.* **27**, 157–162
31. Ingalls, R.G., Squires, R.G., & Butler, L.G. (1975) *Biotech. Bioeng.* **17**, 1627–1637