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SOLID PHASE SYNTHESIS OF A 42-RESIDUE FRAGMENT OF STAPHYLOCOCCAL NUCLEASE: PROPERTIES OF A SEMISYNTHETIC ENZYME

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Abstract.—The polypeptide corresponding to the amino acid sequence from residue 6 through 47 in staphylococcal nuclease has been synthesized by the solid-phase method. The synthetic product closely resembles the corresponding native polypeptide in both physical and chemical properties. The synthetic peptide may be recombined with the complimentary native peptide comprising residues 49 through 149 to form an active, semisynthetic enzyme. The "functional purification" of the crude, synthetic polypeptide by affinity chromatography was found to yield a synthetic fraction of greatly enhanced specific activity. This purification was accomplished on a column of Sepharose to which the complimentary native peptide had been covalently bound.

The major extracellular nuclease of *Staphylococcus aureus* is relatively resistant to the action of proteolytic enzymes, when digestion is carried out in the presence of Ca^{++} and the nucleotide 3′,5′-deoxythymidine diphosphate (pdTp). Under these conditions nuclease may be selectively cleaved by trypsin into an amino-terminal pentapeptide and two inactive, structureless polypeptide fragments termed P_2 (residues 6 through 48) and P_3 (residues 49 through 149). Under appropriate conditions, P_2 and P_3 can recombine through noncovalent interactions to form an active, structurally ordered enzyme derivative, termed nuclease-T.[1, 2] This complex is analogous to the extensively studied ribonuclease-S system.[3, 4] While disulfide bonds play an important role in the structure of ribonuclease-S, such bonds are absent in nuclease-T.

The nuclease-T complex, which possesses about 8% of the activity of native nuclease, provides an excellent model enzyme for the study of the relationships between primary amino acid sequence, three-dimensional conformation, and catalytic activity. Preliminary X-ray diffraction studies of native nuclease indicate that the P_2 and P_3 polypeptide chains occupy opposite hemispheres in the crystalline protein. Both the P_2 and P_3 chains contain residues which appear well situated for interaction with the substrate.[5] The three-dimensional structures of nuclease and nuclease-T have been compared by a number of indirect methods, including heat and urea denaturation, circular dichroism, fluorescence spectroscopy, and tritium exchange.[1, 2, 6] It appears that the structure of nuclease-T is quite similar to that of the native enzyme, but that it is less stable.

The organic synthesis of the P_2 fragment of the nuclease-T complex has been based on the solid phase method developed by Merrifield.[7] This technique has been used by Gutte and Merrifield in the synthesis of an active pancreatic ribo-

nuclease.[8] The synthesis of nuclease fragment P_2 provides another example of the application of the solid-phase method to the reproduction of a large polypeptide sequence. The "functional purification" of the crude polypeptide product by affinity chromatography has been found to be a useful adjunct to this type of synthesis.

Materials and Methods.—N^{α}-*t*-Butyloxycarbonyl (BOC)-L-amino acid derivatives were purchased from either Fox Chemical Co. or Cyclo Chemical Co. and were determined to be of suitable purity by thin-layer chromatography. Side-chain blocking groups included the β or γ benzyl esters (OBzl) of aspartic and glutamic acids, the benzyl ethers (OBzl) of threonine and tyrosine, the N^{ϵ}-trifluoroacetyl (ϵ-TFA) group of lysine, the nitro-guanidino (NO_2) group of arginine, and the imidazole-*N*-carbobenzoxy (im-Cbz) group of histidine. α-BOC-im-Cbz-histidine was synthesized by dissolving BOC histidine (7.5 mmoles) in 25 ml water containing 25 mmoles of $NaHCO_3$. The solution was chilled to 5°C and two 4.5 mmole aliquots of carbobenzoxychloride (Cyclo) were added at 15-min intervals, accompanied by vigorous stirring. After 1 hr, excess CbzCl was removed by ether extraction, and the solution was acidified by addition of 30 mmoles of citric acid. The desired product was extracted with two 25-ml portions of ethyl acetate. After washing with water, the ethyl acetate solution was dried over Na_2SO_4 and flash evaporated to an oil. The oil was dissolved in an appropriate volume of CH_2Cl_2 for addition to the resin in a coupling step. The product showed a major spot, R_f 0.68, on thin-layer chromatography (*n*-butanol, 4: water, 2: pyridine, 1: HAc, 1) with a minor leading contaminant. Staphylococcal nuclease, Foggi strain, and its purified native peptide derivatives, P_2 and P_3, were prepared as previously described.[1, 9]

Coupling procedure: The general scheme of synthesis is shown in Figure 1. It has been shown that treatment of native nuclease-P_2 with carboxypeptidase B will remove lysine residue 48 without loss of activity.[2] Synthesis was therefore begun at proline 47. BOC proline (9 mmoles) was esterified to 10 gm of chloromethylated resin (Cyclo, 1.04 mmoles Cl/gm) in the presence of triethylamine (9 mmoles) by refluxing in ethanol for 52 hr. Synthesis was begun with 5 gm of BOC-prolyl resin, which contained 0.31 mmoles proline/gm. In the repeated coupling cycles the BOC group was removed by treatment for

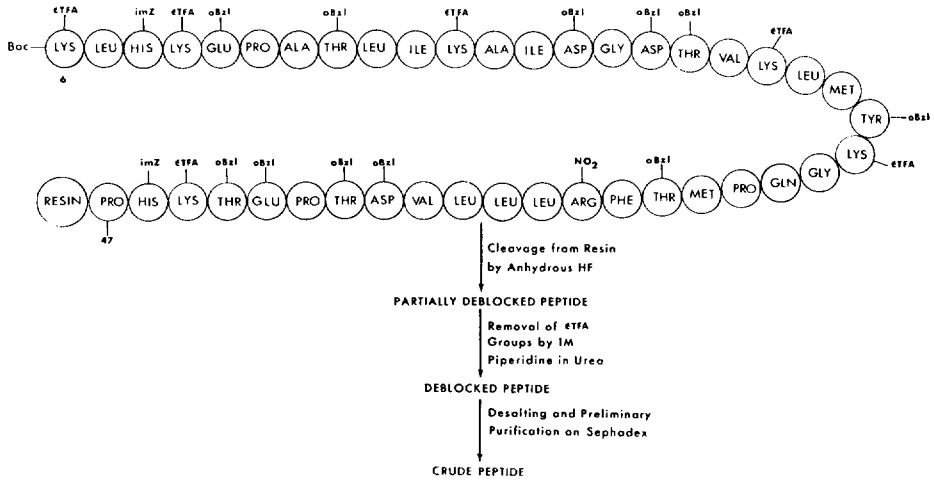

FIG. 1—*Scheme of Solid Phase Synthesis of Nuclease Fragment P_2, Residues 6–47.* Experimental details are given in the text. The amino acid sequence is that of the V_8 strain,[21] with one revision from published data. Glutamic acid, rather than glutamine, has been found in position 43 in both the V_8 and Foggi strains.[22]

1/2 hr with 4 *N* HCl in dioxane. The resulting peptide hydrochloride was converted to the free base by treatment for 10 min with 10% triethylamine in $CHCl_3$. Five millimoles of the appropriate BOC amino acid derivative (threefold excess) was added in a suitable solvent, 5 mmoles of dicyclohexylcarbodiimide (Aldrich) was added, and coupling was allowed to proceed for 2 hr. Glutamine was added as the *p*-nitrophenyl ester in dimethylformamide with a reaction time of 12 hr. Detailed accounts of reagent preparation and rinsing procedures have been published elsewhere.[7, 10] The progress of the synthesis was followed by removal of resin samples after coupling of residue 33 and residue 18. These samples were cleaved from the resin and subjected to amino acid analysis after a standard 20-hr hydrolysis *in vacuo* in 6 *N* HCl at 110°C.

Cleavage procedure: The blocked peptide was cleaved from the resin by treatment with anhydrous HF for 1 hr at 0°C, in the presence of anisole.[11, 12] A typical procedure used 1 gm peptidyl resin, 0.5 ml of anisole, and 10 ml of HF. After removal of the excess HF *in vacuo*, the peptide-resin mixture was extracted 3 times with ethyl acetate to remove remaining anisole, and the ε-TFA peptide was extracted into glacial HAc and lyophilized. One gram of completed peptidyl resin typically yielded 450 mg (0.075 mmoles) of ε-TFA peptide and 400 mg of residual polymer. Since 400 mg of starting polymer contained 0.124 mmoles of the carboxyterminal amino acid, the yield at this stage was approximately 60%.

Removal of ε-TFA groups: The ε-TFA groups were removed by dissolving 100 mg of crude ε-TFA peptide in 5 ml of 1 *M* aqueous piperidine (pH 12.5) and 8 *M* urea for 7 hr at 0°C. This reaction was terminated by application of the mixture to a 2.4 × 100 cm column of Sephadex G-25, followed by elution with 0.05 *M* HAc. The completeness of ε-TFA removal was estimated by the deamination of peptide samples with nitrous acid.[13] The number of intact lysines found by amino acid analysis after this procedure was found to correspond closely to the ε-TFA content in the deblocked peptide as estimated by analysis for elemental fluorine.

Preparation of Sepharose-P_3 column: Native nuclease fragment P_3 was bound covalently to Sepharose (agarose) by the cyanogen bromide activation method.[14] Sepharose 4-B (8 ml bed volume) was suspended by gentle stirring in 20 ml 0.1 *M* $NaHCO_3$ at 5°C and the pH was adjusted to 11 by addition of 2 *N* NaOH. About 150 mg of CNBr (Eastman) was added and stirring continued for 10 min, while pH was maintained at 11 by dropwise addition of 2 *N* NaOH. The suspension was transferred to a chilled sintered glass filter and rinsed quickly with 3 portions each of cold 0.1 *M* $NaHCO_3$, water, and 0.1 *M* phosphate buffer, pH 6.5. This activated Sepharose was stirred gently in 15 ml of phosphate buffer containing 22 mg of fragment P_3 for 3 hr at 5°C. At the end of this time the 280 mμ absorbancy of the supernatant P_3 solution had stabilized at 27% of its starting level, indicating a content of approximately 2 mg of bound P_3/ml of bed volume. The reaction was terminated by exhaustive rinsing of the P_3-Sepharose in a small column with phosphate buffer, water, 0.1 *M* HAc, and water. The column could then be stored indefinitely at 5°C. The relatively low pH of the buffer during the coupling step was found to be important. Coupling of P_3 to the Sepharose proceeded efficiently at pH 9, but the resulting P_3-Sepharose was unable to bind fragment P_2. Coupling at the higher pH probably encourages bond formation at multiple sites on the P_3 molecule, through unionized ε-NH_2 groups. A P_3 molecule bound to Sepharose at several points would not be expected to be capable of free complex formation with P_2.

Results.—Characterization of crude synthetic product: The elution pattern of the deblocked peptide from Sephadex G-25 (Fig. 2) showed a minor peak, beginning at the void volume of the column, and a major retained peak. The ratio of the elution volume of the retained peak to the void volume was similar to that found for native P_2, averaging 1.46. Enzyme generating activity was confined to this fraction. Amino acid molar ratios in an acid hydrolyzed sample of the deblocked and desalted peptide (fraction B) were Lys 6.9 (6), His 2.2 (2), Arg 1.1

(1), Asp 3.2 (3), Thr 4.7 (5), Glu 2.9 (3), Pro 4.6 (4), Gly 2.4 (3), Ala 2.0 (2), Val 1.9 (2), Met 1.8 (2), Ile 2.0 (2), Leu 6.9 (6), Tyr 0.8 (1), Phe 1.1 (1). Theoretical values are given in parenthesis. After nitrous acid deamination, only 0.2 moles of lysine were found, indicating efficient removal of the ϵTFA blocking groups. An enzymatic hydrolysis of the same fraction was performed according to Hill and Schmidt[15] using a combination of pronase, leucine aminopeptidase, and prolidase. Ratios of amino acid were Lys 6.1 (6), His 1.9 (2), Arg 1.2 (1), Asp 3.1 (3), Thr 5.0 (5), Gln 1.1 (1), Glu 2.1 (2), Pro 3.6 (4), Gly 2.7 (2), Ala 2.6 (2), Val 1.9 (2), Met 2.0 (2), Ile 2.1 (2), Leu 6.4 (6), Tyr 0.96 (1), Phe 0.96 (1).

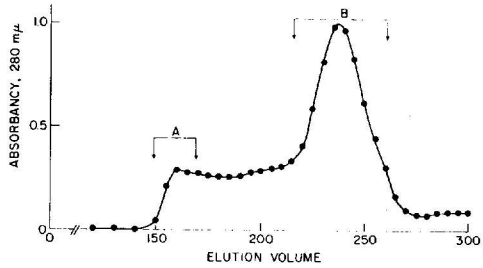

FIG. 2—*Desalting and Preliminary Purification of Crude Synthetic Peptide.* 100 mg of deblocked 6–47 peptide was applied to a 2.4 × 100-cm column of Sephadex G-25 (fine) and eluted with 0.05 *M* HAc. The column void volume is 150 ml.

The partially purified peptide (fraction B) was digested with trypsin in parallel with a sample of native P_2. The fingerprint patterns of the two samples, as shown in Figure 3, were quite similar. Two components present in the synthetic digest but not in the native digest are outlined by dashed lines.

Further purification of crude peptide: Synthetic fraction B was purified further, either by ion exchange or affinity chromatography. Ion-exchange chromatography was carried out on phosphorylated cellulose with a concentration gradient of ammonium acetate. The elution pattern, seen in Figure 4, shows hetero-

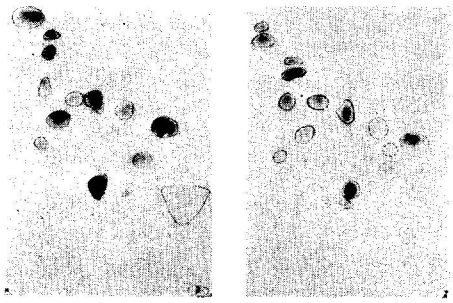

FIG. 3—*Peptide Maps of Tryptic Digests of Native P_2 and Synthetic 6–47 Peptide.* Chromatography was run in *n*-butanol, acetic acid, and water (4:1:5); electrophoresis was run in pyridinium acetate at pH 3.6, 2500 v for 70 minutes.[23] Position of phenol red marker after chromatography is indicated by spot-labeled PR.

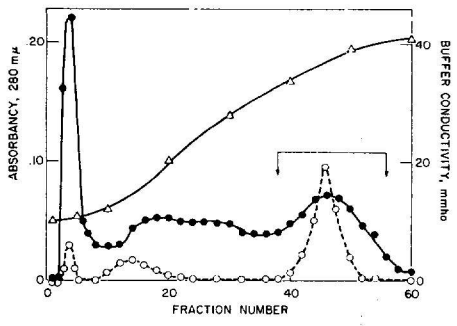

FIG. 4—*Fractionation of Native P_2 and Synthetic 6–47 Peptide on Phosphorylated Cellulose.* 50 mg of 6–47 peptide (fraction *B* from Sephadex G-25) and 10 mg of native P_2 were applied to separate 1.5 × 10 cm columns of phosphorylated cellulose (Whatman) and eluted with a gradient of 100 ml of 0.3 *M* ammonium acetate, pH 6.0, and 100 ml of 1.0 *M* ammonium acetate, pH 8.0. —○, native P_2; —●, synthetic 6–47; —△, buffer conductivity.

geneous behavior. The broad peak corresponding to the typical elution range of native P_2 (35–39 mmho conductivity) was found to possess the highest specific activity. Amino acid analysis of this fraction was not different from that of the crude starting material.

Affinity chromatography was carried out on a 1 × 5 cm column of Sepharose-P_3. In preliminary experiments, binding of small quantities of native P_2 was seen when an excess of P_2 was applied to the column in pH 8 buffer with addition of $CaCl_2$ and pdTp for stabilization of the P_2-P_3 complex. Bound P_2 could be eluted from the column with dilute HAc at pH 3, where fragments P_2 and P_3 are known not to associate. A 5-mg sample of crude synthetic peptide was applied to a similar column and eluted in the same way. As seen in Figure 5, the specific activity of the small bound fraction was approximately 10 times greater than that of the material which failed to bind. This fraction comprised about 7 per cent of the total material applied, and did not differ significantly from the starting material in its amino acid composition. Disk gel electrophoresis of the starting material, the nonbound and the bound peaks showed diminished heterogeneity in the bound peak and a mobility similar to that of

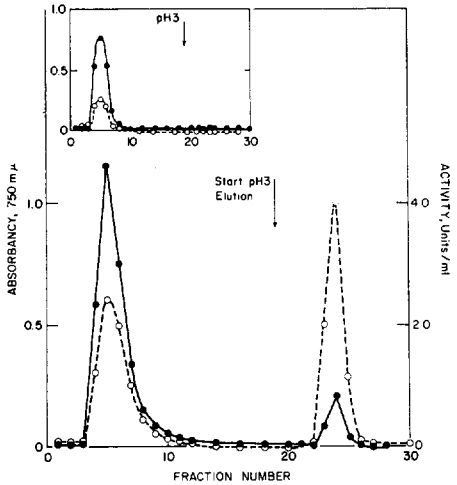

FIG. 5—*Fractionation of Synthetic 6–47 Peptide on Sepharose-P_3.* 5 mg of synthetic peptide was applied to the column in 0.05 *M* borate buffer, pH 8, containing 0.01 *M* $CaCl_2$ and 0.001 *M* pdTp, and eluted with the same buffer. After collection of 19 ml of eluate, the buffer was changed to 0.001 *M* HAc, pH 3. Lowry[24] protein determinations were run on 0.1 ml aliquots of each fraction (—●—). Activities were measured by incubating 0.05 ml of each fraction with 0.05 ml of a 5 mg/ml solution of P_3 in 0.1 *M* Tris, pH 8, and assaying against DNA (–O–). The graph in the upper left corner shows the results of the reapplication of the unbound fraction to the same column.

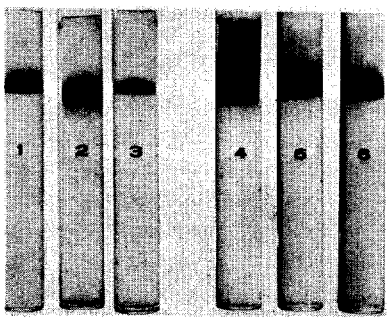

FIG. 6—*Disk Gel Electrophoresis of Native P_2 and Synthetic 6–47 Peptides Before and After Fractionation on Sepharose-P_3.* Polyacrylamide gel electrophoresis (7½% cross-linking) was run at pH 3.3, 3 ma/tube, for 3 hr at 5°C. Direction of migration is toward the anode (bottom of tubes). Tube 1 shows native P_2; tube 2 native P_2 not binding to Sepharose-P_3; tube 3 native P_2 binding to Sepharose-P_3; tube 4 crude 6–47 synthetic peptide; tube 5 synthetic peptide not binding to Sepharose-P_3; tube 6 synthetic peptide binding to Sepharose-P_3.

native P_2 (Fig. 6). When the nonbound synthetic peak was reapplied to the same column, no further binding could be detected. This rejected fraction had a low activity when added to P_3 in free solution but was apparently unable to bind to P_3 on the solid-phase support.

Enzymatic properties of synthetic P_2: The relative specific activities of the crude peptide and of fractions purified by phosphocellulose or Sepharose-P_3 are shown in Figure 7. The DNase activity generated by native P_2 is nearly maximal when it is added to P_3 in a one-to-one molar ratio. The Sepharose-P_3 purified synthetic material, which was the most active of all synthetic derivatives, produced about 30 per cent of full activation when added to P_3 in a one-to-one ratio. When added in a 20- to 40-fold molar excess, material purified by either ion exchange or affinity chromatography could generate 90 per cent of full activity. (Working with larger excesses was difficult because of the limited solubility of both native and synthetic P_2.) Activities measured against RNA (Fig. 8) were

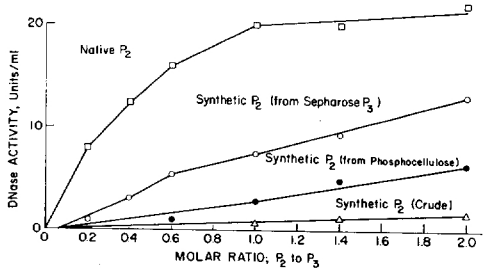

FIG. 7—*DNase Activity Generated by Synthetic P_2 Preparations.* Synthetic P_2 preparations were incubated for 1 hr in varying molar excess with 0.2 mg/ml native P_3 in 0.05 *M* Tris, pH 8, and assayed against DNA by the method of Cuatrecasas *et al.*[25]

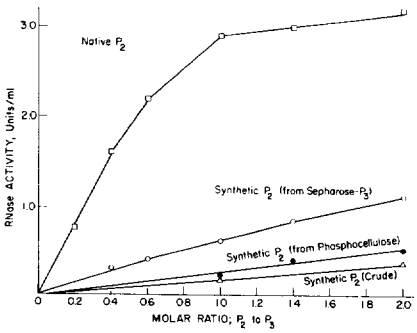

FIG. 8—*RNase Activity Generated by Synthetic P_2 Preparations.* The same solutions shown in Fig. 7 were used.

lower, relative to native P_2. Figure 9 shows the specific activity of control samples of native P_2 after treatment with HF, or with HF followed by piperidine. Successive treatment with both reagents reduced activity by approximately 50 per cent. The conformational stabilities of native and semisynthetic nuclease-T complex were evaluated by trypsin digestion in the presence and absence of Ca^{++} and pdTp (Fig. 10). Both native and semisynthetic complexes showed trypsin resistance only in the presence of metal ion and ligand.

Discussion.—Rationale and limitations in synthetic tactics: Ideal blocking groups for all amino acid side chains in the P_2 sequence have not been found. The im-Cbz group was accepted for histidine even though BOC-im-Cbz histidine is relatively unstable and difficult to purify. In our work BOC histidine (unprotected imidazole) has given unacceptably low coupling efficiency. The ϵ-TFA group for lysine has the disadvantage of requiring rather basic (pH 12.5) conditions for its removal, but has the advantage of complete acid stability. The more frequently used ϵ-Cbz group is removed to a significant extent upon prolonged exposure to the acid conditions of BOC removal.[16] With repeated

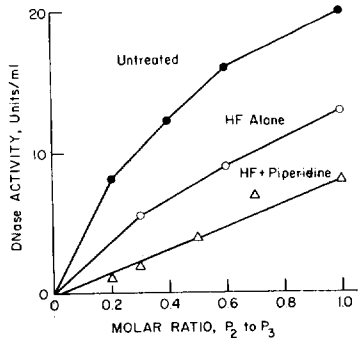

FIG. 9—*DNase Activity of Native P_2 Before and After Treatment with Deblocking Reagents.* Deblocking conditions were the same as those described in the text. After treatment with reagents, the native P_2 samples were desalted on Sephadex G-25, lyophilized, and assayed after incubation with native P_3, as previously described for the synthetic material.

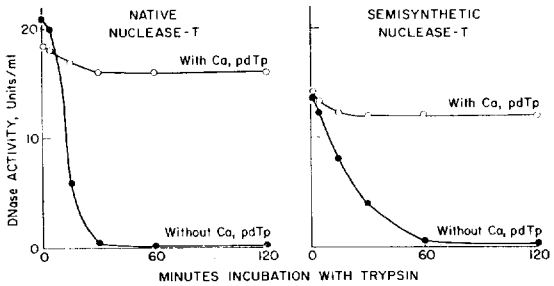

FIG. 10—*Trypsin Inactivation of Native and Semisynthetic Nuclease-T in the Presence and Absence of Ca^{++} and pdTp.* A twofold molar excess of native P_2, or Sepharose-P_3 purified synthetic P_2, was incubated with 0.2 mg/ml P_3 in 0.05 *M* Tris, pH 8, and 0.05 mg/ml DFP-treated trypsin. Concentration of $CaCl_2$ was 0.01 *M* and concentration of pdTp 0.0001 *M* where indicated.

acid exposures in a growing polypeptide chain, there is increasing danger of ϵ-peptidyl side chain formation.[17]

Neither of the deblocking reagents used in P_2 synthesis, HF or piperidine, proved to be innocuous for enzyme activity. The nature of the chemical changes induced in native P_2 is not known. Enzymic hydrolysis of deblocked synthetic material has shown no evidence of racemization or of α-β peptide bond shifts. Fragment P_2 has a tendency to form an inactive aggregate in aqueous solution, particularly at basic pH. Such aggregation may well account for a part of the activity loss observed during deblocking. It is interesting that native nuclease fares somewhat better than fragment P_2 upon exposure to both HF and piperidine, with 70 to 80 per cent of its original activity being recoverable.

Heterogeneity and purification: In large synthetic polypeptides heterogeneity will exist, whatever the means of synthesis. With lengthy solid-phase products only relatively gross errors are detectable by the analytical techniques reported here. A 2 per cent amino acid deletion at each coupling step would pass unnoticed. In such cases it may be desirable to exploit certain functional properties of the synthetic product to improve its purity and specific activity. This principle was used by Hofmann in a purification of ribonuclease-S-peptide.[18] Kato and Anfinsen have recently used a Sepharose-S-peptide column to purify a crude mixture of synthetic S-peptide derivative (residues 1 through 15).[19] With synthetic preparations of complete enzymes, affinity chromatography upon a column bearing a suitable ligand should prove useful. A Sepharose column bearing pdTp has proved very effective in purifying native nuclease.[20]

In the current state of the art, solid-phase synthesis offers a promising technique for studying relationships between structure and function in polypeptides

and proteins of biological interest. It should be emphasized that the problem of heterogeneity, together with low or variable yield of the correct sequence in the synthetic product, dictates caution in the interpretation of results. Nevertheless, valuable information may be obtained when synthetic peptide analogs differ grossly in their biologic activities.

Preliminary studies with synthetic P_2 analogs indicate that residues 6 through 9 (including the histidine residue at position 8) may be deleted without significant loss of binding to P_3 or enzymatic activity. Deletion of residues 6 through 17 leaves an inactive derivative which still binds to a P_3-Sepharose column. Further deletion leads to loss of binding as well. Both methionines in the 6 to 47 sequence may be replaced with norleucine to yield an active analog. Replacement of glutamic acid in position 43 by glutamine leads to complete loss of catalytic activity, while binding to P_3 is retained. A full account of the results of these studies on analogs will be published elsewhere.

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