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Sequences in hydrolysates of thermal poly(glutamic acid, phenylalanine, alanine, methionine)

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The four amino acids glutamic acid, phenylalanine, alanine and methionine were polymerized by heating to a temperature of 190–200°C. Non-random polymers were obtained as evidenced by Edman degradation and FAB mass spectrometry analysis of cyanogen bromide fragments of the polyamino acid polymers. The polymers obtained from the thermal polymerization contained pyroglutamyl *N*-terminal groups, as evidenced by the lack of reaction with ninhydrin reagent. © 1997 Elsevier Science Ltd.

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Introduction

The production of polyamino acids by thermal polymerization of α-aminocarboxylic acids has been carried out by Fox and co-workers[1–3]. In this procedure, mixtures of the amino acids containing glutamic acid, aspartic acid and/or lysine are heated to 120–200°C, and polymers are produced. The time and temperature for the polymerization process are important.

Katchalski[4], among others, found that most amino acids decompose to carbon dioxide, ammonia, amines, carbonyl compounds, etc., on heating. Kovacs *et al.*[5] showed that thermal polyaspartic acid is a polyamide that easily undergoes hydrolysis to produce the α-peptide bond type polyaspartic acids. When Fox and Harada[6] investigated the polymerization of glutamic acid they found that heating did not cause it to polymerize like aspartic acid. However, glutamic acid was found to copolymerize readily. The results of copolymerization are not easily predicted, nor are they as limited as the homopolymerization of aspartic acid. In homopolymerization of aspartic acid a polyaspartic acid polymer is obtained. When glutamic acid is copolymerized with another amino acid, of course, a mixed polymer results. The necessary conditions for polymerization are thus the dry heating of otherwise thermolabile amino acids such as glutamic acid, with one or more of the other amino acids. Lysine can replace the glutamic acid. Without glutamic acid, aspartic acid or lysine in the polymerization mixture, the amino acids decompose rather than polymerize.

The first copolymer synthesized by Fox and Middlebrook[1] involved aspartic acid and leucine in the copolymerization mix. Valine or phenylalanine could be substituted for the leucine. The polyamides formed could be gently hydrolysed to the α-polyamides. Glutamic acid was also copolymerized, but the yields were less than with the aspartic acid[7]. The acidic amino acid, glutamic acid, produced low molecular weight polymers in the range 4000–12 000. Copolymers of lysine with glycine, aspartic acid and glutamic acid have been prepared by Harada[8] with molecular weights in the region of 100 000.

We want to study this polymerization of amino acids in order to understand why in some instances the amino acids are just decomposed at 200°C while glutamic acid, aspartic acid and lysine allow polymerization to occur.

Experimental and results

Preparation of thermal polyamino acids. Mixtures of amino acids as indicated in *Table 1* were heated at 164–195°C under a nitrogen atmosphere from 1 to 17 h to produce the thermally polymerized polyamino acids. A 1-h polymerization time produced a light yellow product, while longer heating times and higher temperatures produced orange and brown–black products. All of the products readily dissolved in acetone/water (9/1). The reaction products were all biuret positive, indicating the presence of peptide bonds in the products.

Analysis of thermal polyamino acids by paper chromatography. Whatman 3MM paper was used for the separation of the polyamino acids in the various reaction products. The two solvent systems most commonly used were BAW (n-butanol/acetic acid/water) (5/1/4, v/v) and BPAW (n-butanol/acetic acid/water/pyridine) (15/3/12/10). *Figure 1* indicates a one-dimensional paper chromatogram of thermal polyamino acids that was allowed to run for 17 h. The chromatogram was allowed to dry and developed with chlorox, ethanol and then starch–iodide (1% of each). The chromatogram indicates quite different results for the 1 to 17 h polymerization times. Two spots are observed for the 1 h polymer and five spots for the 17 h polymer.

In *Figure 2* the 17 h polymer S_4 was chromatographed on Whatman 3MM paper and sprayed with starch–iodide reagent on the left side, indicating positions of thermal polyamino acids, and with ninhydrin on the right side, indicating positions of free amino acids.

The four starch–iodide spots were eluted with acetic acid/water (9/1). The separate acetic acid eluates were

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Table 1 Names and description of the prepared thermal polyamino acids

Names	Temp. (°C)	Polymerization time (h)	Starting material (moles)			
			Glu	Ala	Phe	Met
S1	164	3	0.017	0.005	0.003	0.003
S2	161	17	0.017	0.005	0.003	0.003
S4	194	17	0.017	0.005	0.003	0.003
S6	190	17	0.017	0.005	0.003	0.003
S7	195	17	0.017	0.005	0.003	0.006
S8	195	1	0.017	0.005	0.003	0.003

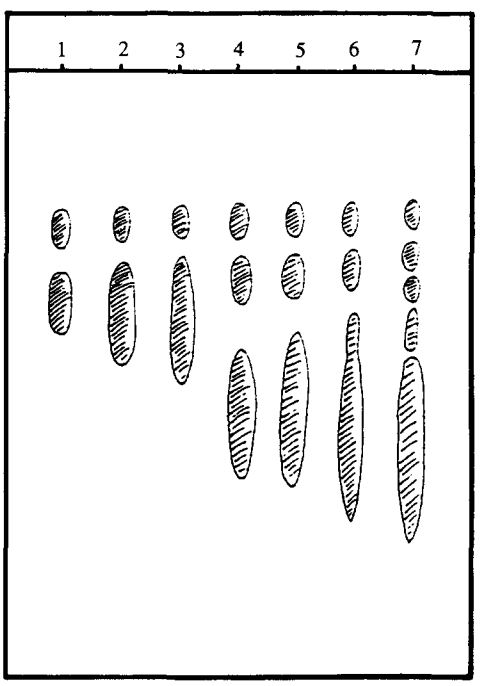

Figure 1 One-dimensional paper chromatography of thermal polyamino acid mixture. The paper was developed with BAW for 17 h and sprayed with starch–iodide: (1) 1 h polymer; (2) 2 h polymer; (3) 3 h polymer; (4) 4 h polymer; (5) 5 h polymer; (6) 6 h polymer; (7) 17 h polymer

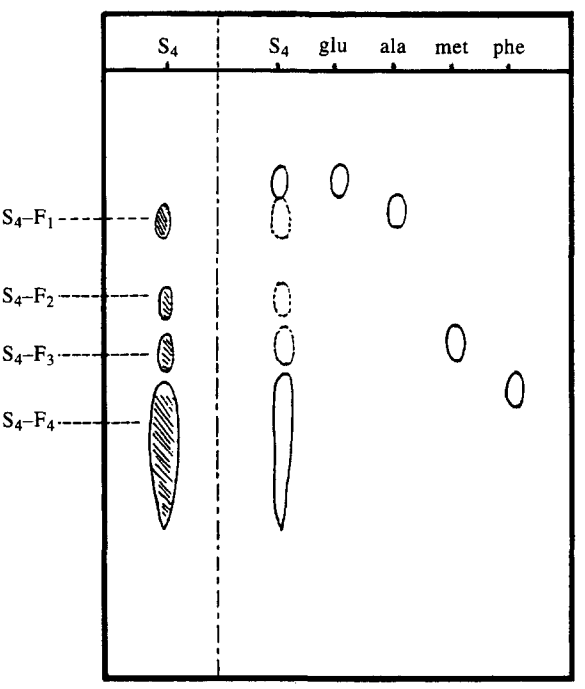

Figure 2 One-dimensional paper chromatography of 17 h polymer (S_4). Left side of the paper was sprayed with starch–iodide and the right side with ninhydrin. Shaded spots, dark colour; open spots, light colour

dried and then hydrolysed with 6 M hydrochloric acid. The hydrolysates were then subjected to analysis on a Beckman automatic amino acid analyser. The S_4–F_4 (highest R_f number) contained Glu, Ala, Phe and Met, while the S_4–F_3 spot contained Glu, Ala and Phe.

Figure 3 shows the results for the polymers (S_7) formed with twice as much Met present in the polymerization mixture. Three starch–iodide spots were found. Again, the spot with the greater R_f value contained Glu, Ala, Phe and Met. The Met containing fractions S_4–F_4 and S_7–F_3 were obtained from reaction mixtures heated at 194–195°C for 17 h. Some ninhydrin positive fractions were unreacted amino acids. None of the thermal polyamino acids were ninhydrin positive.

For the S_7 polymers, the amino acid composition of the various fractions (S_7–F_1, S_7–F_2 and S_7–F_3) are summarized in *Table 2*.

Cyanogen bromide treatment S_4–F_4 fraction to obtain S_4–F_4–CN1 fraction. The S_4–F_4 fraction was dissolved in 0.1 N HCl, and a 50-fold excess of crystallized cyanogen bromide was added. The reaction proceeded for 24 h at room temperature in a reactivial. The mixture was diluted 10-fold with water, and dried on a rotary

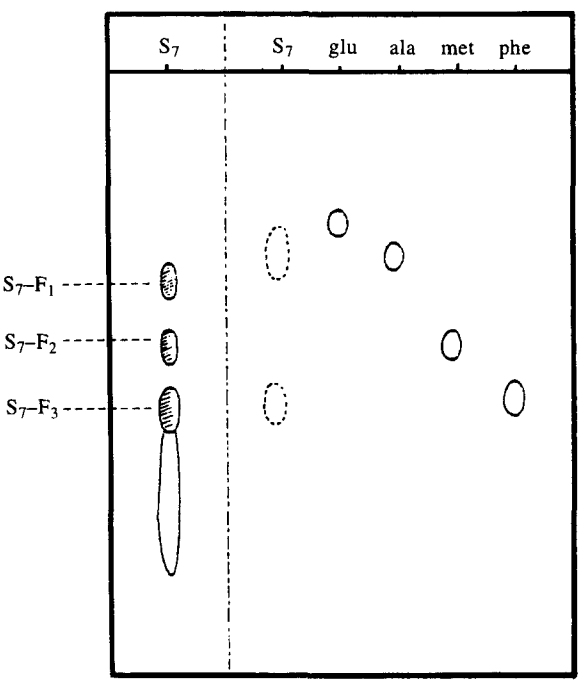

Figure 3 One-dimensional paper chromatography of S_7 polymer. Left side of the paper was sprayed with starch–iodide and the right side with ninhydrin. Shaded spots, dark colour; open spots, light colour

evaporator. Upon paper chromatography, the digest produced five starch–iodide spots and four ninhydrin staining spots. The starch–iodide spot with the lowest R_f value was identified as the S_4–F_4–CN1 fraction.

Amino acid sequence determination by Edman degradation. A tightly stoppered Kimax tube was used for carrying out the entire sequence of reactions. A 200 μl sample of the peptide was dissolved in 2 ml of a buffer containing *N*-ethylmorpholine (60 ml), acetic acid (1.5 ml) and ethanol (500 ml) made up to 1 l with distilled water. Phenylisothiocyanate (0.1 ml) was then added to 2 ml of the S_4–F_4–CN1, and the reaction proceeded for 2.5 h at 40°C. The solution was then evaporated to dryness under reduced pressure and extracted twice with benzene.

The cyclization reaction was carried out with 2 ml of anhydrous trifluoroacetic acid at 25°C for 1 h. The trifluoroacetic acid was then removed by evaporation and 4 ml of 0.2 M acetic acid was added. The solution was extracted with benzene, and the aqueous layer was dried under nitrogen. The dried material was dissolved in 0.05 M ammonium acetate and 50% acetonitrile, pH 6.8.

Table 2 Amino acid composition of thermal polyamino acid fraction S_7–F_1, S_7–F_2 and S_7–F_3

	Fraction (mol%)[a]		
Amino acid	S_7–F_1[b]	S_7–F_2	S_7–F_3[c]
Glu	43.5	78.8	45.6
Ala	56.5	19.2	35.8
Phe	—	—	2.3
Met	—	—	16.2

[a] Analysis by h.p.l.c. on Alltech C_{18} column with 0.1% phosphoric acid (pH 2.5), flow rate 1 ml min^{-1}; 20°C
[b] Fastest moving fraction on column elution
[c] Slowest moving fraction on column elution

The solution was filtered through a 0.3 μm Millipore filter before analysis by high performance liquid chromatography (h.p.l.c.). The h.p.l.c. was performed on a Spectra Physics h.p.l.c. system with an Alltech C_{18} column (10 μm; 25 cm × 4.6 mm). The buffer used was 0.05 M ammonium acetate and 50% acetonitrile at 50°C, with the detector wavelength set at 254 nm. The flow rate was 1.0 ml min^{-1} with a pressure of 1000 psi. All four phenylthiohydantoin (PTH) derivatives were well separated.

The stepwide degradation of S_4–F_4 cyanogen bromide fractions is shown in *Table 3*. We can see, e.g. in fraction S_4–F_4–CN1, that Glu and Ala appear in all six positions starting from the *N*-terminus of the polyamino acid, which is evidence that there is more than one polymer in that fraction. The same appears true for the other four fractions: S_4–F_4–CN2, S_4–F_4–CN3, S_4–F_4–CN4 and S_4–F_4–CN5, with CN3, CN4 and CN5 also containing Phe. Of course, all the Met has been converted into homoserine, which is at the *C*-terminus of only those polyamino acid polymer fragments formed from the cyanogen bromide reaction. All these polymer fragments are thus larger than hexapeptides.

Fast atom bombardment mass spectrometry (FAB m.s.) of thermal polyamino acids. The technique of FAB m.s. was developed by Vickerman *et al.*[9]. The thermal polyamino acids were dried completely and thioglycerol was added. The mass spectra were recorded on a VG 70 E mass spectrometer equipped with a VG-11-250 data station.

The positive and negative ion mass spectra of the thermal polyamino acid fragments were obtained. The technique used has allowed the spectra to be obtained without conversion of the peptide into volatile derivatives. The data show that the production of gaseous negative ions from the thermal polyamino acid (S_4–F_4–CN1) was promoted by the presence of acidic Glu

Table 3 Edman degradation of thermal polyamino acid (S_4–F_4) after cyanogen bromide treatment

		Concentration of PTH–amino acid (nmol/0.2 ml)					
Sample	PTH–AA[a]	1	2	3	4	5	6
S_4–F_4–CN1[b]	PTH–Glu	1.0	1.0	1.0	1.0	1.0	1.5
	PTH–Ala	1.5	3.2	3.8	4.6	3.2	1.0
	Total	2.5	4.2	4.8	5.6	4.2	2.5
S_4–F_4–CN2	PTH–Glu	1.0	2.1	1.0	1.0	1.0	1.0
	PTH–Ala	0.0	1.0	4.5	3.6	3.8	4.4
	Total	1.0	3.1	5.5	4.6	4.8	5.4
S_4–F_4–CN3	PTH–Glu	2.9	1.8	1.0	1.0	1.6	1.0
	PTH–Ala	1.0	2.3	2.4	6.5	2.0	2.8
	PTH–Phe	0.0	1.0	2.0	2.6	1.0	0.0
	Total	3.9	5.1	5.4	10.1	4.6	3.8
S_4–F_4–CN4	PTH–Glu	1.3	1.4	1.0	1.0	1.0	2.4
	PTH–Ala	1.0	2.2	1.5	2.6	1.2	2.7
	PTH–Phe	0.0	1.0	1.7	0.0	1.2	1.0
	Total	2.3	4.6	4.2	3.6	3.4	6.1
S_4–F_4–CN5	PTH–Glu	2.3	1.5	1.0	1.4	1.9	3.0
	PTH–Ala	1.0	1.8	1.6	6.8	2.7	1.0
	PTH–Phe	0.0	1.0	1.8	1.0	1.0	2.3
	Total	3.3	4.3	4.4	9.2	5.6	6.3

[a] PTH–AA phenylthiohydantoin amino acid derivative obtained from Edman process
[b] S_4–F_4–CN are cyanogen bromide cleavage fragments of polyamino acid

Table 4 Fragments of thermally produced polyamino acid (SF_4–F_4–CN1)

No.	*m/z*	Fragment	Relative intensity (%)
1	663	Ala_4, Glu_2, Hsr	11
2	590	Ala_3, Glu_2, Hsr	12
3	462	Ala_3, Glu, Hsr	39
4	332	Ala_3, Hsr	23
5	189	Ala, Hsr	22
6	376	Glu_2, Hsr	50
7	274	Glu_2	41

residues. The negative ion fragments found are listed in *Table 4*. From the table, both Glu–Hsr and Ala–Hsr appear in the original thermal polymer. The predicted probable sequences of the original thermal polymer preparation are:

1. Glu–Ala–Glu–Ala–Ala–Hsr
2. Ala–Ala–Glu–Glu–Ala–Ala–Hsr
3. Glu–Ala–Ala–Glu–Ala–Ala–Hsr
4. Ala–Glu–Glu–Ala–Ala–Ala–Hsr
5. Ala–Ala–Ala–Ala–Glu–Glu–Hsr

Sequence ions of these polyamino acids were found. It is interesting that the bulk of the fragmentation arises from the *N*-terminal end of the molecule. This was previously observed by MacFarlane and Tongerson[10]. The finding of 50% relative intensity for the Glu_2, Hsr ($m/z = 376$) fragment in *Table 4* indicates that thermal polymer sequence no. 5 occurs in greatest quantity in thermal polymer fraction S_4–F_4–CN1. The fragment no. 3 ($m/z = 462$) containing Ala_3, Glu, Hsr with relative intensity 39% shows that no. 4 must occur in the second greatest quantity in fraction S_4–F_4–CN1. Upon closer inspection, nos 1 and 3 may also give rise to fragment (m/z 462) Ala_3, Glu, Hsr. Thus, S_4–F_4–CN1 may contain at least four different polymers. Thus, the results of the FAB m.s. experiment agrees with the sequence determinations by Edman degradation of the S_4–F_4–CN1 fraction that the Glu and Ala occur in each position, which indicates that there is more than one thermal polyamino acid in S_4–F_4–CN1. The Ala–Ala–Ala–Ala–Glu–Glu–Hsr no. 5 sequence is the most abundant, as we can observe from *Table 4* where no. 6 ($m/z = 376$) fragment with a Glu–Glu–Hsr peptide occurs in 50% relative intensity. The next most abundant peptide is no. 3 with m/z of 462 and relative intensity of 39%. Thus, of the vast number of thermal peptide sequences possible, two, nos 3 and 5, containing polyamino acids, make up the bulk of thermal polymers formed in the polymerization process.

Discussion

The thermal polymerization of amino acid mixtures is a dehydration reaction in which lactams are produced if glutamic acid is present[11,12]. Phillips and Melius[13] detected pyroglutamic acid (a lactam) in a heated amino acid mixture used to produce thermal polyamino acids. In the experiments reported here, Glu, Ala, Phe and Met were converted into polyamino acids by heating for up to 17 h at 161–195°C. The polymers were ninhydrin negative, indicating pyroglutamyl *N*-termini. These polymers were subjected to mild alkaline hydrolysis to cleave the *N*-terminal pyrrolidone rings of pyroglutamyl groups, which then allowed analysis by the Edman procedure.

Fox and Nakashima[11] had studied the polymerization of Glu, Tyr and Gly by heating in the dry state to 180°C. They were able to identify pyroGlu–Gly–Tyr and pyroGlu–Tyr–Gly as major peptide products. Other di- or tri-peptides were not observed. Hartmann *et al.*[12] established that the reactions occurred by first conversion of glutamic acid into pyroglutamic acid, which then reacted with the diketopiperazine, glycyl–tyrosine, to form the two tripeptides pyroGlu–Gly–Tyr and pyroGlu–Tyr–Gly.

In the experiments performed in this work, Met was incorporated into the thermal polyamino acids only at the high temperatures of 194 and 195°C and 17 h of heating. When the reaction mixtures were chromatographed on Whatman 3MM paper using a butanol, acetic acid, water solvent, three fractions were obtained. The fastest moving fraction contained Met when reaction times were 10 h or more at 195°C. Phenylalamine was incorporated after 3 h of heating and alanine after 1 h of heating.

The S_4–F_4–CN2 fragment in *Table 3* contains only Glu at the *N*-terminus, which indicates that it is the *N*-terminal cyanogen bromide fragment of S_4–F_4. The other fragments CN1, CN3, CN4 and CN5 contain Glu and Ala at the *N*-terminus, but not Phe. Thus, there are no Met–Phe sequences in the original polyamino acid. If we had completely sequenced the cyanogen bromide fragments the one with no homoserine would of course have been the *C*-terminal peptide fragment.

The fragment no. 6 from FAB m.s. analysis, as given in *Table 4*, shows that the major fragment has a Glu–Glu–Hsr sequence at the *C*-terminus. Fragments nos 7, 1 and 2 are all consistent with fragment no. 6. Thus, the sequence of the *C*-terminus of the major polyamino acid in S_4–F_4–CN1 is:

Ala–Ala–Ala–Ala–Glu–Glu–Met(Hsr) (50%)

The minor fragment is (from nos 3 and 4) Glu–Ala–Ala–Ala–Met(Hsr) (39%). Thus, the polymerization of the Glu, Phe, Met and Ala in these reactions is non-random.

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References

1. Fox, S. W. and Middlebrook, M., *Federation Proc.*, 1954, **13**, 211.
2. Harada, K. and Fox, S. W., *Arch. Biochem. Biophys.*, 1960, **86**, 274.
3. Fox, S. W., Harada, K. and Vegotsky, A., *Experimentia*, 1959, **15**, 81.
4. Katchalski, E., *Adv. Protein Chem.*, 1951, **6**, 123.
5. Kovacs, J., Koenyves, F. and Pusztai, A., *Experimentia*, 1953, **9**, 459.
6. Fox, S. W. and Harada, K., *Science*, 1958, **128**, 1214.
7. Harada, K. and Fox, S. W., *J. Am. Chem. Soc.*, 1958, **80**, 2694.
8. Harada, K., *Bull. Chem. Soc. Jpn.*, 1959, **32**, 1008.
9. Vickerman, J. C., Bordoli, R. S. and Barber, M., *J. C. S., Chem. Comm.*, 1981, **7**, 324.
10. MacFarlane, R. D. and Tongerson, D. F., *Science*, 1976, **191**, 920.
11. Fox, S. W. and Nakashima, T., *Biochim. Biophys. Acta.*, 1967, **140**, 155.
12. Hartmann, J., Brand, M. C. and Dose, K., *BioSystems*, 1981, **13**, 141.
13. Phillips, R. D. and Melius, P., *Int. J. Pept. Protein Res.*, 1974, **6**, 309.