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Note

Separation and identification of ribonuclease S-peptide methyl esters by ion-exchange high-performance liquid chromatography and ^{1}H nuclear magnetic resonance spectroscopy

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The isolated ribonuclease fragment 1–19 (S-peptide) has been shown to form in aqueous solution at low temperature an α-helix structure similar to that found in the ribonuclease-S crystal[1,2], which is an indication that this fragment can act as a nucleation centre in the folding of the protein. In order to identify the interactions stabilizing the helix, it is of great interest to examine the folding properties of S-peptide analogues[3,4], in particular those having the carboxylate groups in a modified form, since they are potential candidates for involvement in salt bridges. After isolating the $O^{\delta,2}$ methyl S-peptide by means of low-pressure ion-exchange chromatography[3], we thought that, by using both stronger conditions in the esterification reaction and a separating system of higher resolution[5,6], other S-peptide methyl esters could be obtained.

In this note the obtention, separation and identification of four S-peptide diesters are reported for the first time. Separation was achieved by semipreparative ion-exchange high-performance liquid chromatography (HPLC) and peak identification by ^{1}H NMR spectroscopy.

EXPERIMENTAL

Apparatus and reagents

A liquid chromatograph (Knauer, Bad Homburg, F.R.G.) equipped with two pumps, a programmer, injector (Rheodyne, 7125) and an UV detector set at 220 nm was used. The fraction collector was from LKB and the freeze dryer from Virtis. Solvents, HPLC grade, were from Scharlau; ammonium acetate was from Panreac and water was obtained from a Millipore Milli-Q system.

S-peptide (Sigma Type XII-A, Lot 102-F8020) was purified by passage through a 25 cm × 1.6 cm I.D. preparative reversed-phase column (Knauer; Polygosil C_{18}, d_p = 7.5 μm). Injections of 15 mg peptide dissolved in 0.5 ml of water were made. Flow-rate: 5 ml/min. Elution profile: 0–5 min, 14% A in B, isocratic; 5–35 min, linear gradient from 14 to 18% A in B. Solvents: A, acetonitrile–water (70:30) with 0.02%

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trifluoroacetic acid (TFA); B, water with 0.02% TFA. Under such conditions two major peaks were partially resolved which correspond to a mixture of the ribonuclease 1–20 and 1–19 peptides, in an approximate intensity ratio of 1:3, as previously reported[7].

Obtention of S-peptide esters

The esterification reaction was performed following standard methods[8]. Approximately 20 mg of purified S-peptide were suspended in 2.3 ml methanol, and then hydrochloric acid was added to a final concentration of 0.1 *M*. The reaction tube was kept at 25°C in a thermostat bath for 24–30 h. Aliquots of the reaction mixture were periodically examined by ion-exchange HPLC to check the intensity of the peaks corresponding to the S-peptide esters. When they reached maximum intensity, 1 ml of water was added and the reaction stopped by cooling the tube in a bath of solid carbon dioxide–acetone, while simultaneously freeze-drying until a dry product was obtained. This was redissolved in 0.5 ml of water, filtered through HV-0.45 μm Millipore membranes and then chromatographed.

Ion-exchange separation of the esters

A weak anion-exchange column, Synchropack AX-100 (Synchrom Inc., Linden, IN, U.S.A.), 25 cm × 0.4 cm I.D., d_p = 5 μm, pore size 100 Å, was employed in both analytical and semipreparative runs. Gradient profile: 0–1 min, 75% A in B; 1–31 min, linear gradient from 75 to 30% A in B. Solvents: A, acetonitrile; B, ammonium acetate 10 m*M*, pH 6.0). Flow-rates of 2 ml/min and 20-μl injections were used. The conditions for preparative runs were the same, except that the injection volumes and gradient time were 250 μl (≈10 mg peptide) and 60 min respectively. The solvent system employing triethylammonium acetate[6] at pH 4.4 was also tested and gave slightly better resolution than ammonium acetate; however, the latter was preferred for preparative purposes, because it is readily eliminated during the freeze-drying process, and the residual acetate gives only one NMR signal, thus minimizing overlapping.

^1H NMR spectra

Spectra were recorded at 360 MHz on a Bruker WM-360 pulse spectrometer using conditions reported elsewhere[7]. Samples were prepared by dissolving lyophilized fractions directly in ^2H_2O (Stholer, 99.8%). Saturation of the ^2HOH signal and approximately 3000 scans were necessary to obtain an adequate signal-to-noise ratio.

RESULTS AND DISCUSSION

Fig. 1a shows an analytical chromatogram taken at the first stages of the reaction where, apart from the S-peptide (peak G), only peak F corresponding to the $O^{\delta,2}$ methyl monoester appears intense. As the reaction proceeds, peaks A, B and C, corresponding to S-peptide diesters as shown below, increase, reach a maximum and then begin to decrease (Fig. 1b). The formation of S-peptide triesters that are no longer retained in the column accounts for the decrease. Such triesters are highly unstable, since the fractions corresponding to the void volume, when dissolved in water and chromatographed again, reproduce the same pattern of peaks, A to G,

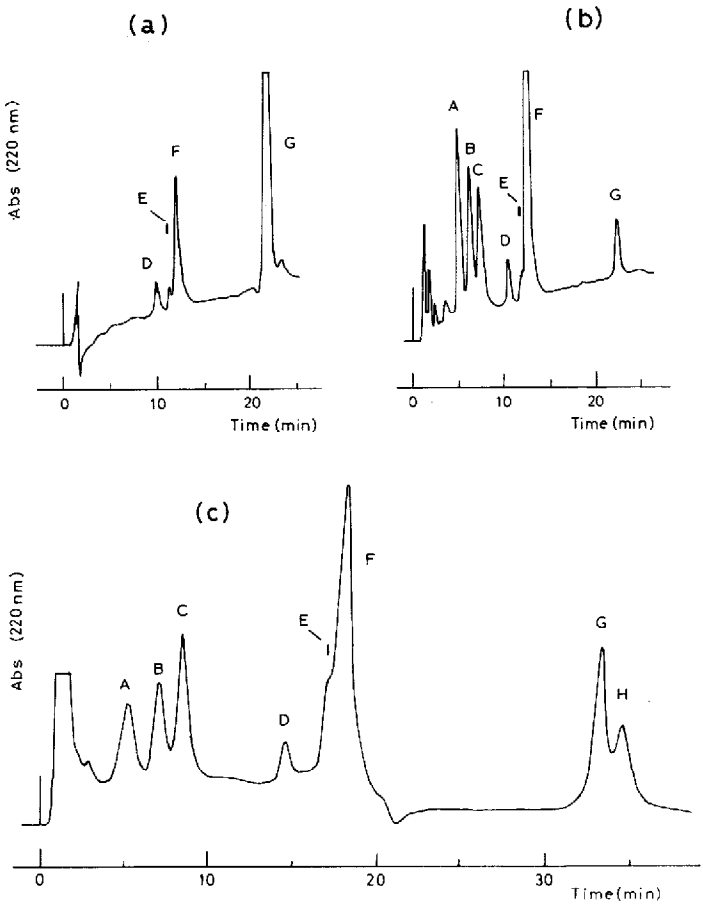

Fig. 1. Ion-exchange HPLC chromatograms showing the time course of the esterification reaction of S-peptide (see Experimental for conditions): (a) after 2 h; (b) after 20 h; (c) preparative separation after completion of the reaction (30 h).

although with different intensities. No attempt has been made to separate such highly unstable triesters.

Fig. 1c shows the preparative chromatogram of the mixture of esters at the point of maximum diester formation. No significant loss of resolution with respect to the analytical separations was observed, with the volumes and substance loads used. Fractions corresponding to the eight different peaks detected in the chromatogram were collected, lyophilized and redissolved in ^2H_2O. Selected regions of their ^1H NMR spectra taken at pH ≈ 7 are shown in Fig. 2. Such regions, corresponding to the H_α signal of Ala 19, $H_{\gamma\gamma'}$ signals of Glu 2, Glu 9 and Gln 11 and $H_{\beta\beta'}$ signal of Asp 14 residues, are the most favourable ones to detect low-field δ changes upon formation of methyl esters in the adjacent carboxylate groups. The region 3.6–3.8 ppm, where $-OCH_3$ ester resonances appear, is also shown in Fig. 2 with the vertical scale reduced by a factor of four. Assignments of the ^1H NMR spectrum of the

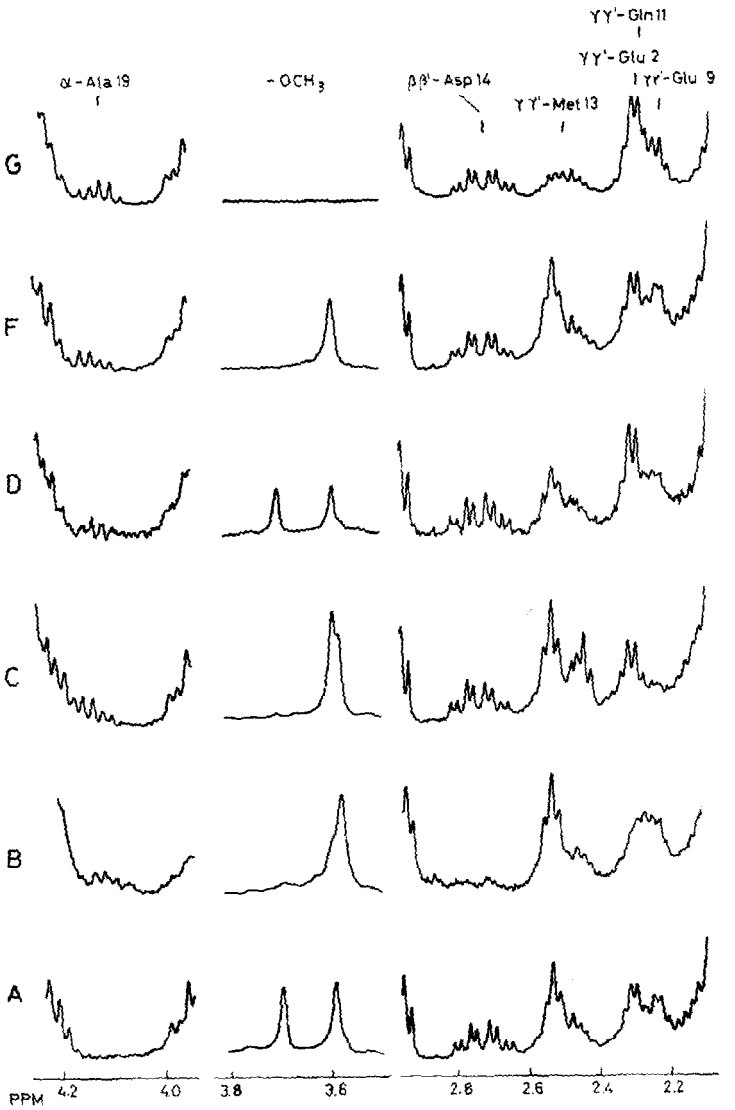

Fig. 2. Selected regions of the ^1H NMR spectra (^2H_2O, pH 7) of the peaks collected from the preparative chromatogram shown in Fig. 1c. Assignments are given for the spectrum of the unmodified S-peptide (peak G).

unmodified S-peptide given in Fig. 2 are readily obtained from published δ values and pH titration shifts[7].

The identification of peaks A, B, C and F was straightforward from the number of $-OCH_3$ resonances in the spectra and the type of NMR signals that shifted downfield with respect to their positions for the unmodified S-peptide (see Table I and Fig. 2). Due to the shortage of substance, only the spectrum of peak F was seen in the fraction corresponding to shoulder E. Peak H, which increased only in the latest stages of the esterification process, was very unstable providing, when recorded, an

TABLE I

NMR IDENTIFICATION OF VARIOUS S-PEPTIDE ESTERS

*Peak**	*Shifting NMR signals*	*No. of $-OCH_3$ groups*	*Identification*
A	$\delta\delta'$ Glu 2; α Ala 19	2	$O^{\delta,2}$-Methyl, S-peptide methyl ester
B	$\delta\delta'$ Glu 2; $\beta\beta'$ Asp 14	2	$O^{\delta,2}$-Methyl, $O^{\gamma,14}$-methyl-S-peptide
C	$\delta\delta'$ Glu 2; $\delta\delta'$ Glu 9	2	$O^{\delta,2}$-Methyl, $O^{\delta,9}$-methyl-S-peptide
D	$\delta\delta'$ Gln 11; α Ala 19	2	$O^{\delta,11}$-Methyl, S-peptide methyl ester
E	–	–	(Not enough substance)
F	$\delta\delta'$ Glu 2	1	$O^{\delta,2}$ Methyl-S-peptide
G	None	None	Unmodified S-peptide
H	–	–	(unstable) S-Peptide when examined

* Labelling corresponds to that of Fig. 1c.

spectrum identical to that of the unmodified S-peptide. In the spectrum of peak D the intensities of the $-OCH_3$ resonances were seen to decrease with time, finally yielding S-peptide. In such a mixture the identification is difficult, but it is clear that the carboxylates of residues 9 and 14 are not modified, while that of the 19th residue is. Peak D is certainly a diester and the additional esterified carboxylate cannot correspond to Glu 2, because the 2–19 diester had a different retention time, so it must correspond to the Gln 11 residue, which is also susceptible to esterification[8]. This conclusion is in accord with the intensity changes detected in the $H_{\delta\delta'}$ region of the spectrum, and also with the position of peak D in the chromatogram, close to that of the $O^{\delta,2}$ monoester (note that the esterification of Gln 11 does not modify the charge of the molecule).

From the results given here, it appears that weak anion-exchange HPLC[5,6] will greatly facilitate the obtention of selectively modified carboxylate groups on other polypeptides of biological interest. Helix-forming properties of the S-peptide esters isolated here are now being studied.

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