A Revised Primary Structure of Neocarzinostatin Using the Combination of Endopeptidase, Carboxypeptidase Y and Fast Atom Bombardment Mass Spectrometry

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The amino acid sequence of antitumor protein neocarzinostatin was revised and confirmed on the basis of mass spectrometric studies. Fast atom bombardment mass spectrometric experiments on neocarzinostatin and its tryptic, chymotryptic, staphylococcal protease, and carboxypeptidase Y digested peptides gave the molecular weights of various peptides and, in some cases, partial sequence information. These experiments gave a revised sequence, Glu(105)-Pro(106) to Pro(105)-Glu(106) and Asp(60) to Asn(60). The peptide fragments produced by these endopeptidase cleavage completely covered the full sequence of neocarzinostatin. Thirty-five of the 113 amino acids were determined by the carboxypeptidase Y digestion.

Mass spectrometric techniques have been used a number of times in the determination of the primary structure of peptides and proteins. Fragmentation of the electron impact method has been used for a long time in this area. Over the last year, new ionization techniques, field desorption (FD) mass spectrometry and fast atom bombardment (FAB) mass spectrometry, have been developed. These are paticularly suitable for underivatized peptides producing rather abundant molecular ions (M^+) or protonated molecular ions (MH^+). It is difficult to determine the primary structure of peptides by mass spectrometry alone. Shimonishi et al. established the combination of FD or FAB mass spectrometry with Edman degradation or exopeptidase digestion.[1-10] These techniques are suitable for peptide mixtures and may prove to be a valuable approach technique for the sequence determination of peptides and proteins.

Neocarzinostatin is an antitumor antibiotic protein[11-15] isolated from culture filtrates of *Streptomyces carzinostaticus*.[11] It is a single-chain polypeptide constituted of 113 amino acids and exists as an apoprotein and an associated nonprotein chromophore. The amino acid sequence of neocarzinostatin was first reported in 1972 by Maeda et al. using the Edman degradation method.[16,17] The structure of neocarzinostatin was corrected in 1982 by Biemann by a combination of tryptic and chymotryptic digestion, FABMS and GCMS (Fig. 1).[18] Recently, we found that there were a number of peptides from staphylococcal protease digests with m/z that conformed to the published sequence (Fig. 1), indicating the correctness of these regions, and two (m/z 706.9 and 2753.3) that did not. This clearly shows that the sequence is in error. Therefore, we reinvestigated the previously proposed primary structure of neocarzinostatin by a combination of endopeptidase, carboxypeptidase Y and FABMS without the Edman degradation method. Consequently, we found that Glu(105)-Pro(106) must be corrected to Pro(105)-Glu(106) and Asp(60) must be replaced with Asn(60) in Fig. 1. Recently, Biemann and coworkers published the revised primary structure of neocarzinostatin, in which Glu(105)-Pro(106) was corrected to Pro(105)-Glu(106) by our indication and by their GCMS data.[19] In addition, the structure of the chromophore was determined in 1985.[20]

We report in this paper the revised primary structure of neocarzinostatin and the reason for the exchange of Glu(105)-Pro(106) for Pro(105)-Glu(106)

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                                   10                                  20
Ala-Ala-Pro-Thr-Ala-Thr-Val-Thr-Pro-Ser-Ser-Gly-Leu-Ser-Asp-Gly-Thr-Val-Val-Lys-

                                   30                                  40
Val-Ala-Gly-Ala-Gly-Leu-Gln-Ala-Gly-Thr-Ala-Tyr-Asp-Val-Gly-Gln-Cys-Ala-Trp-Val-

*                                  50                                  60
Asp-Thr-Gly-Val-Leu-Ala-Cys-Asp-Pro-Ala-Asn-Phe-Ser-Ser-Val-Thr-Ala-Asp-Ala-Asp-

                                   70                                  80
Gly-Ser-Ala-Ser-Thr-Ser-Leu-Thr-Val-Arg-Arg-Ser-Phe-Glu-Gly-Phe-Leu-Phe-Asp-Gly-

                                   90                                 100
Thr-Arg-Trp-Gly-Thr-Val-Asn-Cys-Thr-Thr-Ala-Ala-Cys-Gln-Val-Gly-Leu-Ser-Asp-Ala-

                                  110
Ala-Gly-Asp-Gly-Glu-Pro-Gly-Val-Ala-Ile-Ser-Phe-Asn
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Fig. 1. The primary structure of neocarzinostatin.
* Asn of original report seems to be a misprint.

and the replacement Asp(60) with Asn(60) by the combination of the enzymatic cleavage (trypsin, chymotrypsin, staphylococcal protease), carboxypeptidase Y digestion and FABMS.

Experimental

Mass Spectrometry. Positive ion FAB mass spectra were recorded with a JMS HX-100 double focusing mass spectrometer fitted with a 23 kilogauss high field magnet, operating at accelerating voltages at 5 kV. The atom emission gun current of 20 mA was maintained at 6 kV for the generation of the xenon fast atom beam. Mass spectra were recorded with a JMA-3500 mass data system. A sample of the underivatized peptides was prepared by dissolving c. 5 μg samples in c. 1.5 μl of 1:1 glycerol–1 M† HCl. This solution was deposited on a stainless steel sample stage for introduction into the mass spectrometer ion source on the end of an axially mounted sample introduction probe.

Reagents and Enzymes. Dithiothreitol and iodoacetic acid were obtained from Wako Pure Chemical Industries Ltd., (Japan). Staphylococcal protease was purchased from Miles Laboratories Inc., (U.S.A.). Trypsin and chymotrypsin were from Sigma Chemical Co., (U.S.A.) and carboxypeptidase Y from Oriental Yeast Co., Ltd. (Japan).

Reduced and Carboxymethylated Neocarzinostatin. Commercially available neocarzinostatin (Yamanouchi Seiyaku Co., Inc.) was dialyzed against water and lyophilized. The crude neocarzinostatin was washed with methanol several times, dialyzed against water and lyophilized. Five μmol of neocarzinostatin was dissolved in 6 ml of Tris/HCl buffer (pH 8.0) containing 8 M urea and 0.2% EDTA. After addition of 300 μmol of dithiothreitol, the mixture was gassed with nitrogen for 10 min before heating for 3 h at 50 °C and was allowed to stand overnight in the dark at room temperature. To this mixture 100 mg of iodoacetic acid in 0.6 ml of 1 M NaOH were added. Alkylation was conducted at pH 8.0 or 8.5 by addition of 1 M NaOH. After 30 min the mixture was dialyzed against 0.2 M acetic acid and lyophilized.

Enzymatic Digestion (a) Trypsin: Under the standard conditions of 50:1 molar ratio of substrate to trypsin in 50 μl of 0.2 M ammonium acetate buffer (pH 8.0), 0.5 mg of the reduced and carboxymethylated neocarzinostatin was digested. The solution was incubated at 25 °C for 6 h. The hydrolysis was stopped by freezing and lyophilizing the solution.

(b) Chymotrypsin: The reduced and carboxymethylated neocarzinostatin (0.5 mg) or its endopeptidase digested peptides (0.5 mg) were treated under the conditions of 200:1 molar ratio of substrate to chymotrypsin in 50 μl of 0.2 M ammonium acetate buffer (pH 8.0). The solution was incubated at 25 °C for 30 min. The hydrolysis was stopped by freezing and lyophilising the solution.

(c) Staphylococcal Protease: The reduced and carboxymethylated neocarzinostatin (0.5 mg) was digested under the conditions of a 50:1 molar ratio of substrate to staphylococcal protease in 50 μl of 5 mM ammonium carbonate buffer (pH 8.8). The solution was incubated at 37 °C for 24 h. The hydrolysis was stopped by freezing and lyophilising the solution.

(d) Carboxypeptidase Y: After digestion by endopeptidase, the subpeptides were lyophilised and added to 100 μl of 0.05% pyridine–acetate buffer (pH 6.0) containing 20 μg of carboxypeptidase Y. The enzyme digestion was carried out at 37 °C, and aliquots were removed from the reaction mixture at different time intervals (1 min, 10 min, 1 h, 2 h) and isolated by freeze-drying.

Results and Discussion

(a) Chymotryptic Peptide: The positive ion FAB mass spectrum of the chymotryptic digest of the reduced and carboxymethylated neocarzinostatin indicated the presence of at least seven peptides shown in Fig. 2. Each peptide appears as the corresponding protonated molecular ions, MH^+. Five of the seven chymotryptic peptides (C1, C2, C5, C6, and C7) fit the published sequence on the basis of chymotrypsin specificity. The peptides C3 and C4 did not fit any fragment expected from the enzyme specificity (Table 1). If either Asp(57) or Asp(60) is replaced by Asn, C3 and C4 are matched to the position Ala(46)–Leu(67) and Ser(53)–Leu(67) in Fig. 1, respectively, on the basis of their m/z and chymotrypsin specificity.

(b) Tryptic Peptide: Only two of the four expected peptides could be found in the positive ion FAB mass spectrum of the tryptic peptides of the reduced and carboxymethylated neocarzinostatin; those with m/z=1859.0 (T1) and 1275.5 (T2) (data not shown). The other two peptides could not be found because they were too large to be observed by the spectrometer. The two tryptic peptides were matched with the sequence shown in Fig. 1 on the basis of

† 1 M=1 mol dm^{-3}.

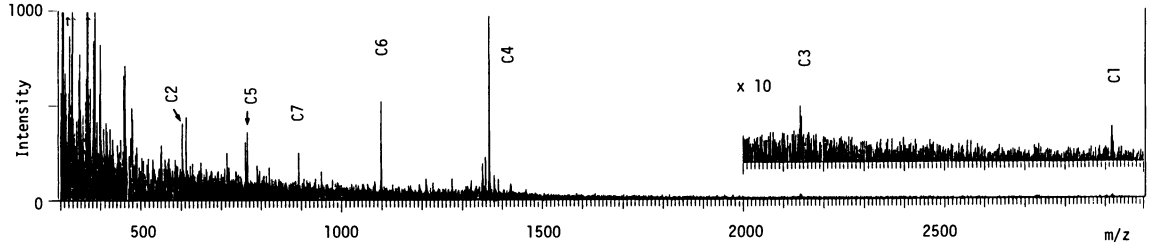

Fig. 2. The positive ion FAB mass spectrum of the chymotryptic digest of the reduced and carboxymethylated neocarzinostatin.

Table 1. Peptides Generated by Enzymatic Cleavage of the Reduced and Carboxymethylated Neocarzinostatin

Peptide	Observed mass value	Theoretical[a] mass value	Matching section (based on enzyme specificity) of:	
			Structure shown in Fig. 1.	Structure shown in Fig. 7.
C1	2918.8	2918.50	Ala (1)-Tyr (32)	Ala (1)-Tyr (32)
C2	603.3	603.33	Val (40)-Leu (45)	Val (40)-Leu (45)
C3	2144.0	2143.90	None	Ala (46)-Leu (67)
C4	1367.6	1367.62	None	Ser (53)-Leu (67)
C5	765.2	765.43	Thr (68)-Phe (73)	Thr (68)-Phe (73)
C6	1098.4	1098.56	Thr (68)-Phe (76)	Thr (68)-Phe (76)
C7	894.4	894.44	Leu (77)-Trp (83)	Leu (77)-Trp (83)
T1	1859.0	1858.97	Ala (1)-Lys (20)	Ala (1)-Lys (20)
T2	1275.5	1275.59	Ser (72)-Arg (82)	Ser (72)-Arg (82)
ST1	1374.5	1374.67	Ala (1)-Asp (15)	Ala (1)-Asp (15)
ST2	503.7	503.31	Gly (16)-Lys (20)	Gly (16)-Lys (20)
ST3	1193.2	1193.59	Val (21)-Asp (33)	Val (21)-Asp (33)
ST4	935.3	935.39	Val (34)-Asp (41)	Val (34)-Asp (41)
ST5	1725.8	1725.76	Thr (42)-Asp (58)	Thr (42)-Asp (58)
ST6	1163.3	1163.59	None	Ala (59)-Arg (70)
ST7	598.8	598.28	Gly (75)-Asp (79)	Gly (75)-Asp (79)
ST8	2439.5	2439.00	None	Trp (83)-Glu (106)
ST9	707.1	707.36	None	Gly (107)-Asn (113)
SC1	1374.6	1374.67	Ala (1)-Asp (15)	Ala (1)-Asp (15)
SC2	1677.7	1677.87	Gly (16)-Asp (33)	Gly (16)-Asp (33)
SC3	935.3	935.39	Val (34)-Asp (41)	Val (34)-Asp (41)
SC4	1165.3	1165.51	Thr (42)-Phe (52)	Thr (42)-Phe (52)
SC5	1367.4	1367.62	None	Ser (53)-Leu (67)
SC6	1007.5	1007.56	Leu (67)-Glu (74)	Leu (67)-Glu (74)
SC7	598.8	598.28	Gly (75)-Asp (79)	Gly (75)-Asp (79)
SC8	2753.1	2753.17	None	Gly (80)-Glu (106)
SC9	707.1	707.36	None	Gly (107)-Asn (113)

a) Calculated from the revised sequence.

their trypsin specificity (Table 1).

In order to determine the amino acid sequence of the tryptic peptides, they were subjected to chymotryptic cleavage and carboxypeptidase Y digestion. Figure 3 shows the positive ion FAB mass spectra of the chymotryptic peptides and of the digest with carboxypeptidase Y. The chymotryptic subpeptides TC1, TC2, TC3, TC5, TC6, and TC7 fit the sequence on the basis of their trypsin and chymotrypsin specificity. The peptide TC4 did not conform to enzyme specificity. After treatment with carboxypeptidase Y, subpeptide TC1(m/z 1858.6) gave mass peaks at m/z 1631.7, m/z 1532.8, and m/z 1431.5, on the FAB mass spectra. The results indicated that the C-terminal sequence of TC1 was -Thr-Val-[Val, Lys] (see Table 2). Similarly, the mass peaks at m/z 915.5, m/z 844.2, m/z 743.2, m/z 686.2, and m/z 615.0 from the subpeptide TC2 (m/z 1078.1) indicated that the C-terminal sequence of TC2 was -Ala-Gly-Thr-Ala-Tyr. In the same way, the mass peaks at m/z 490.2 from the subpeptide TC3 (m/z 603.7) showed that the C-terminus of TC3 was -Leu (or Ile). In the same manner, the mass peaks at m/z 1254.3, m/z 1167.5, m/z 1066.3, m/z 979.3, m/z 908.3, m/z 821.1, m/z 650.1, and m/z 579.6 from the subpeptide TC4 (m/z 1367.2) indicated that the C-terminal sequence of TC4 was -Ala-[Asn, Gly]-Ser-Ala-Ser-Thr-Ser-Leu(or Ile). If 60th amino acid residue is Asp, these mass peaks must be m/z 1255, m/z 1168, m/z 1067, m/z 980, m/z 909, m/z 822, m/z 650, and m/z 579. Accordingly it was necessary to replace Asp(60) with Asn in Fig. 1. Furthermore, the mass peaks at m/z 2666.9, m/z 2496.0, m/z 2439.1, and m/z 2309.8 from the subpeptide TC7(m/z 3126.9) indicated that the C-terminal sequence of TC7 was -Glu-Gly-[Val, Ala]-[Ile(or Leu), Ser, Phe, Asn]. These results show that the 106th amino acid residue of the sequence must be Glu.

(c) Staphylococcal Protease Digested Peptides: Two FABMS experiments were carried out on the staphylococcal protease digested peptides. First, the staphylococcal protease digested peptides were hydro-

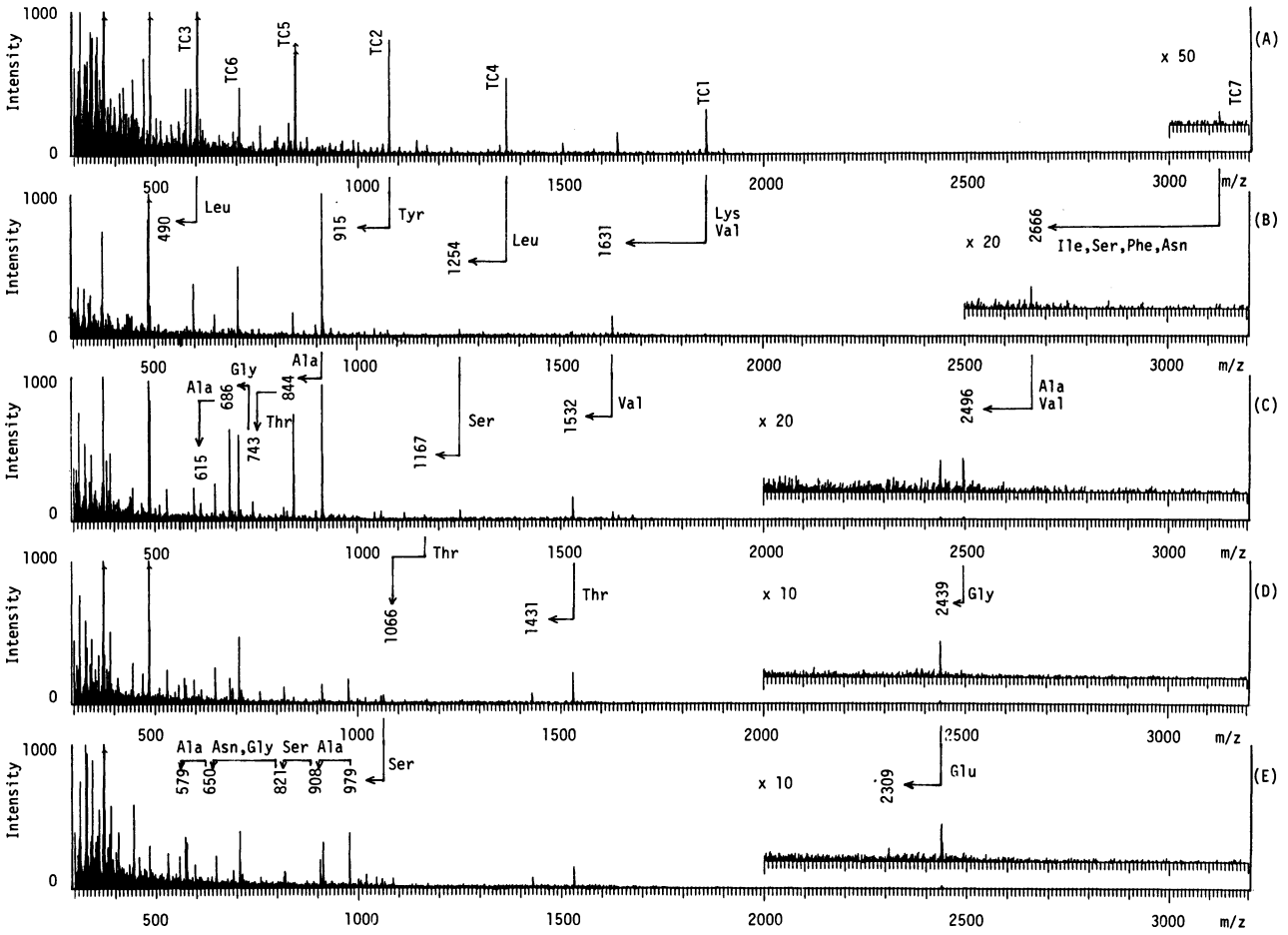

Fig. 3. The positive ion FAB mass spectra of the chymotryptic digest of the tryptic subpeptides (A) and of the digest with carboxypeptidase Y (B: 1 min, C: 10 min, D: 1 h, E: 2 h).

Table 2. Peptides Generated by Chymotryptic Cleavage of the Tryptic Peptides and of the Digest with Carboxypeptidase Y

Peptide	Observed mass value	Theoretical mass value	Matching section		C-terminal sequence
			Fig. 1.	Fig. 7.	
TC1	1858.6	1858.97	1—20	1—20	-Thr-Val-[Val, Lys]
TC2	1078.1	1078.55	21—32	21—32	-Ala-Gly-Thr-Ala-Tyr
TC3	603.7	603.33	40—45	40—45	-Leu(or Ile)
TC4	1367.2	1367.62	None	53—67	-Ala-[Asn,Gly]-Ser-Ala-Ser-Thr-Ser -Leu(or Ile)
TC5	846.9	846.40	72—78	72—78	
TC6	708.8	708.36	77—82	77—82	
TC7	3126.9	3127.35	None	83—113	-Glu-Gly-[Val, Ala]-[Ile(or Leu), Ser, Phe, Asn]

lyzed with trypsin or chymotrypsin, and the total hydrolysate was subjected to FABMS to give the molecular weights of the resulting peptides. Second, to determine the C-terminal sequence of staphylococcal protease digested peptides, they were subjected to carboxypeptidase Y digestion. The positive ion FAB mass spectrum of the tryptic peptides is shown in Fig. 4. Six tryptic peptides ST1, ST2, ST3, ST4, ST5, and ST7 fit the sequence shown in Fig. 1 on the basis of staphylococcal protease and trypsin specificity (Table 1). But the peptides ST6, ST8, and ST9 could not be matched with the published sequence on the basis of enzyme specificity.

If Asp(60) is replaced by Asn, ST6 fits the position Ala(59)-Arg(70) in Fig. 1 on the basis of its *m/z* and enzyme specificity. If Glu(105)-Pro(106) is Pro(105)-

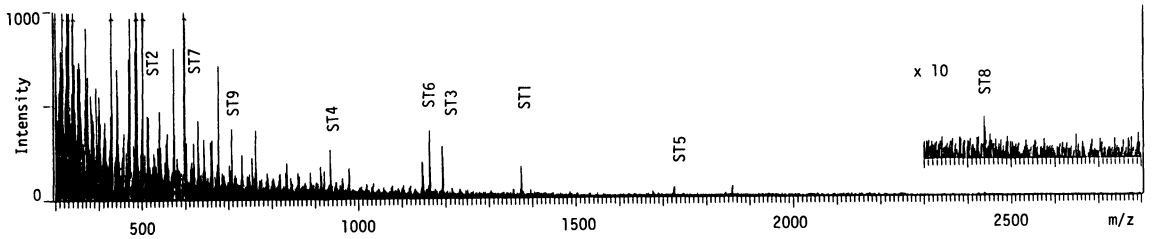

Fig. 4. The positive ion FAB mass spectrum of the tryptic digest of the staphyococcal protease digested subpeptides.

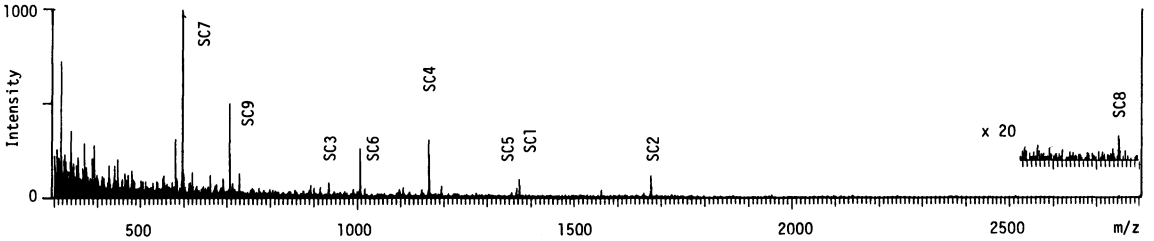

Fig. 5. The positive ion FAB mass spectrum of the chymotryptic digest of the staphylococcal protease digested subpeptides.

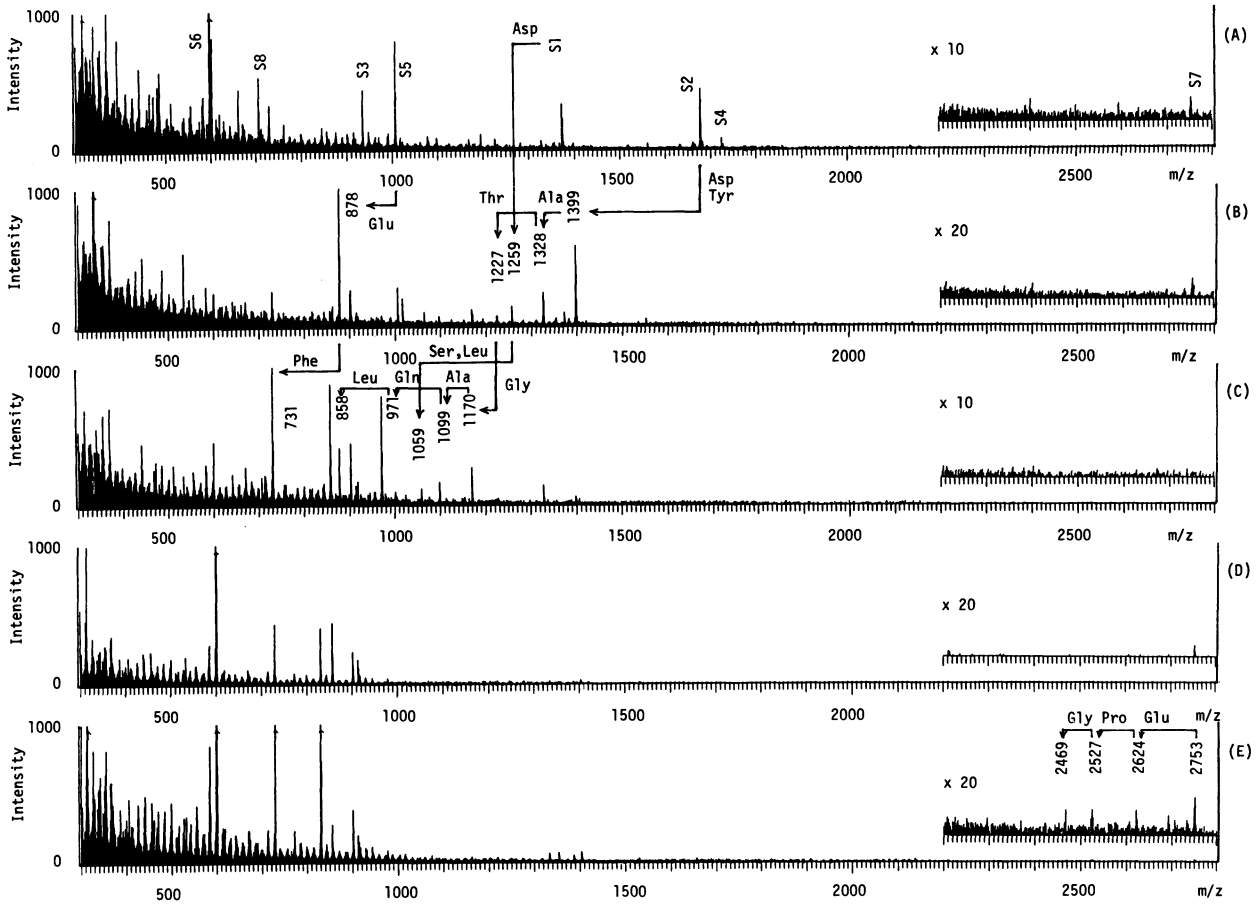

Fig. 6. The positive ion FAB mass spectra of the staphylococcal protease digested peptides of the reduced and carboxymethylated neocarzinostatin (A) and of the digest with carboxypeptidase Y (B: 1 min, C: 10 min, D: 1 h, E: 2 h).

Glu(106), ST8 and ST9 are matched to the position Trp(83)–Glu(106) and Gly(107)–Asn(113), respectively.

The positive ion FAB mass spectrum of the chymotryptic digest is shown in Fig. 5. Six chymotryptic peptides SC1, SC2, SC3, SC4, SC6, and SC7 fit the published sequence on the basis of staphylococcal protease and chymotrypsin specificity (see Table 1). The peptides SC5, SC8, and SC9 did not correspond to any fragment expected from the enzyme specificity.

If either Asp(57) or Asp(60) is replaced by Asn, SC5 corresponds to the position Ser(53)–Leu(67) in Fig. 1 on the basis of their *m/z* and enzyme specificity. If Glu(105)–Pro(106) is Pro(105)–Glu(106), SC8 and SC9 are matched to the position Gly(80)–Glu(106) and Gly(107)–Asn(113), respectively.

Figure 6 shows the positive ion FAB mass spectra of the staphylococcal protease digested peptides of the reduced and carboxymethylated neocarzinostatin and of their digest with carboxypeptidase Y. Six subpeptides S1, S2, S3, S4, S5, and S6 fit the published sequence on the basis of staphylococcal protease specificity. But the peptides S7 and S8 did not correspond to any fragment expected from the enzyme specificity (Table 3).

After treatment with carboxypeptidase Y, peptide S1 (*m/z* 1374.0) gave the mass peaks at *m/z* 1259.6

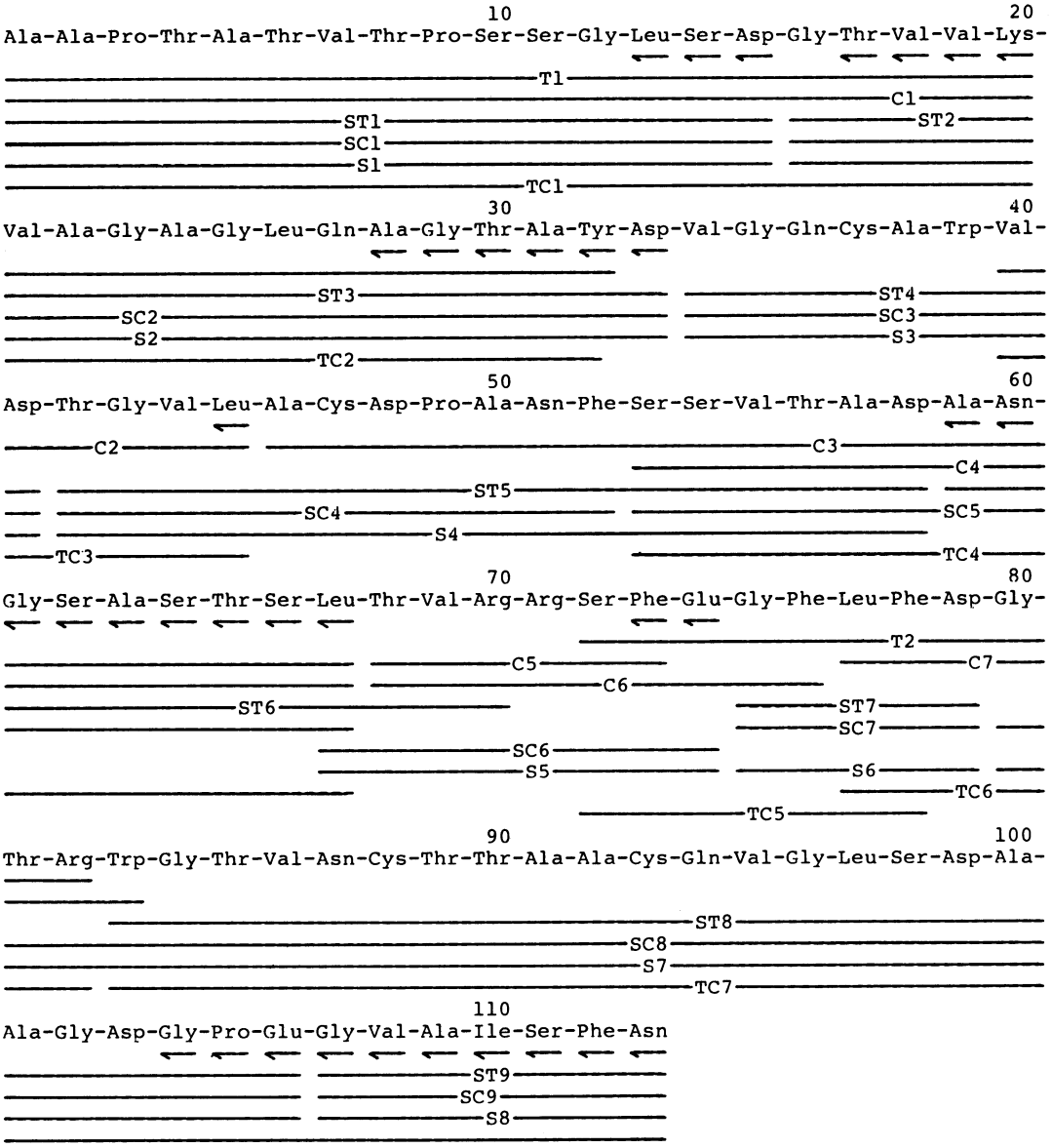

Fig. 7. The correct primary structure of neocarzinostatin and the sequence data with the results of endopeptidase and carboxypeptidase Y. ←—, Carboxypeptidase Y data; FABMS peptides produced by cleavage with trypsin (T), chymotrypsin (C), staphylococcal protease (S), staphylococcal protease and trypsin (ST), staphylococcal protease and chymotrypsin (SC), and trypsin and chymotrypsin (TC) are underlined.

Table 3. Peptides Generated by Staphylococcal Protease Cleavage of the Reduced and Carboxymethylated Neocarzinostatin and of the Digest with Carboxypeptidase Y

Peptide	Observed mass value	Theoretical mass value	Matching section		C-terminal sequence
			Fig. 1.	Fig. 7.	
S1	1374.0	1374.67	1—15	1—15	-[Leu(or Ile), Ser]-Asp
S2	1677.4	1677.87	16—33	16—33	-Leu-Gln(or Lys)-Ala-Gly-Thr-Ala-Tyr-Asp
S3	935.0	935.39	34—41	34—41	
S4	1725.3	1725.76	42—58	42—58	
S5	1007.1	1007.56	67—74	67—74	-Phe-Glu
S6	598.7	598.28	75—79	75—79	
S7	2753.3	2753.17	None	80—106	-Gly-Pro-Glu
S8	706.9	707.36	None	107—113	

and *m/z* 1059.5 on the FAB mass spectra. The results indicated that the C-terminal sequence of S1 was [Leu(or Ile), Ser] -Asp (see Table 3). Similarly, the mass peaks at *m/z* 1399.8, *m/z* 1328.8, *m/z* 1227.7, *m/z* 1170.6, *m/z* 1099.6, *m/z* 971.5 and *m/z* 858.3 from the peptide S2 (*m/z* 1677.4) indicated that the C-terminal sequence of S2 was -Leu-Gln(or Lys)-Ala-Gly-Thr-Ala-Tyr-Asp. In the same manner, the mass peaks at *m/z* 878.7 and *m/z* 731.2 from the peptide S5(*m/z* 1007.1) indicated that the C-terminal sequence of S5 was -Phe-Glu. Similarly, the mass peaks at *m/z* 2624.4, *m/z* 2527.1 and *m/z* 2469.8 from the peptide S7(*m/z* 2753.3) showed that the C-terminal sequence of S7 was -Gly-Pro-Glu. These results show that the position of Glu(105) and Pro(106) must be interchanged with each other.

The correct primary structure of neocarzinostatin and the sequence data are summarized in Fig. 7 with the results of carboxypeptidase Y digestion. This primary structure is the same as shown in Fig. 1 except Glu(105)-Pro(106) and Asp(60). Asp(60) would be caused by a variety of neocarzinostatin or by a mutation of *Streptomyces carzinostaticus* or by chemical change when it is isolated. Consequently, the subpeptides C3, C4, ST6, ST8, ST9, SC5, SC8, SC9, TC4, TC7, S7, and S8 correspond to the position 46—67, 53—67, 59—70, 83—106, 107—113, 53—67, 80—106, 107—113, 53—67, 83—113, 80—106, and 107—113 in Fig. 7. As shown in Fig. 7, the peptide fragments produced by the three kinds of enzymatic digestion completely covered the full sequence of neocarzinostatin. This implied the usefulness of the combination of enzymatic digestion and FAB mass spectrometry for the primary sequence studies of proteins.

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