CHARACTERIZATION OF THE AMINO TERMINI OF MOUSE SALIVARY AND PANCREATIC AMYLASES

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Received 12 February 1981

1. Introduction

Genetic and biochemical studies of salivary and pancreatic amylases from a variety of animals suggest that the two amylases arose as the result of gene duplication (reviewed in [1]). The hypothesis has been confirmed for the mouse amylases by the sequencing of cDNAs produced from mRNAs of the two enzymes [2]. The amino acid sequence deduced from the base sequence of the mRNA for each amylase is expected to contain the amino acid sequence of a signal peptide along with that of the secreted enzyme [3]. The secreted mammalian amylases studied to date have blocked N-termini [1], none of which have as yet been characterized with respect to the amino acid which is blocked. As a consequence, it was not possible to determine from the mRNA data where the sequences of the secreted enzymes begin.

We have used the mouse amylase mRNA sequences [2] and information concerning cleavage of signal sequences from other proteins [4] to locate potential cleavage points for removal of the signal peptides from the mouse amylases. That in turn allowed us to predict the biochemical characteristics of the N-terminal tryptic peptide for each enzyme and then use those characteristics to locate and purify the peptide. Analysis of the N-terminal peptides showed that the secreted mouse amylases have pyroglutamic (pyrrolidone carboxylic) acid residues at their N-termini.

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2. Materials and methods

Salivary and pancreatic amylases were purified from parotid glands and pancreases, respectively, taken from mice of strain C3H/As [5]. Prior to sacrifice and dissection, the mice were starved for 4 h, a treatment which optimizes the amylase yield. Parotid glands and, in separate preparations, pancreases were homogenized in 3 vol. 0.05 M potassium phosphate buffer (pH 6.9) containing 7 mM NaCl and 1 mM phenylmethylsulfonylfluoride (PMSF). Following homogenization in an Ultra-Turrax TP18-10 (Ika Werk) homogenizer, 1 ml 1% Triton X-100 was added/10 ml homogenate. The homogenates were then mixed gently by hand, allowed to incubate 30 min at room temperature (22°C) and then stored frozen for 18 h at −20°C. The homogenates were thawed and centrifuged for 20 min at 12 000 × *g*. Amylase was purified from each supernatant by a scaled-up version of the cycloheptaamylose affinity chromatography method [6] as modified [5]. Amylase purity was determined by electrophoresis in polyacrylamide gels containing sodium dodecylsulfate (SDS) [5].

Separation and detection of peptides by high-voltage paper electrophoresis, column chromatography methods for the separation of peptides and amino acids, and determination of N-termini by dansylation were as in [7]. A specific color test for peptides containing histidine and/or tyrosine [8] was also used.

Mouse salivary amylase (3.2 μmol) was dissolved in 100 ml 0.3 M Tris–HCl (pH 8.2) containing 8 M urea and reduced for 60 min with dithiothreitol (DTT). Iodoacetic acid was then added and carboxymethylation allowed to proceed for 30 min. The mixture was

desalted on a Sephadex G-25 (Pharmacia) column (5 × 50 cm) in 0.1 M NH_4HCO_3 and the void volume peak fractions pooled. An aliquot (100 nmol) of the reduced, carboxymethylated protein was stored frozen for N-terminal analysis. The amylase was digested with 1/100 (w/w) trypsin (Worthington) for 3 h at 37°C in the elution buffer. The reaction was stopped by the addition of soybean trypsin inhibitor (Sigma) and lyophilized. The pancreatic amylase was similarly derivatized and digested with trypsin. Tryptic digests of the amylases were chromatographed on 2.5 × 110 cm columns of Sephadex G-50 in 0.1 M NH_4HCO_3.

The putative N-terminal peptide produced by trypsin digestion of salivary amylase was subdigested with chymotrypsin (Worthington) and the corrresponding peptide from pancreatic amylase was subdigested with elastase (Worthington). In each case the peptide was treated with 1/100 (w/w) of the proteolytic enzyme for 3 h at 37°C in 0.1 M NH_4HCO_3 buffer (pH 8.3). The samples were then subjected to two-dimensional electrophoresis in which the first dimension was run 20 min at pH 6.5 and the second dimension was run 20 min at pH 2.1 [7]. The position of the N-terminal peptide was calculated, in each case, from the predicted amino acid composition based on the expected cleavage site. Other peptides were visualized by fluorescamine staining [7]. Peptides were cut from the two-dimensional maps, eluted from the paper with 1% pyridine and analyzed for amino acid composition and N-termini [7].

Mass spectra were recorded on a Varian Mat 311 A mass spectrometer using direct introduction of the samples. The resolution was 1000 and the electron energy 70 eV. Peptides were deuteroacetylated (CH_3OH:$(C^2H_3-\overset{O}{\overset{\|}{C}})_2O$ 3:1, 30 min) and permethylated (CH_3I) for 1 min [9] using *t*-butylate, dimethylsulfoxide [10] as the base.

3. Results and discussion

Putative amino acid sequences for mouse salivary and pancreatic amylases have been generated from cDNA [2]. Those amino acid sequences are expected to contain the signal peptides for the two enzymes along with the amino acid sequences of the secreted enzymes. The proteolytic process which removes the signal peptides from secreted proteins [11] appears to act preferentially at residues with side chains containing at most a single carbon moiety [4]. That, combined with the observation that mammalian amylases studied to date have blocked N-termini [1], suggests that such a processing site in the putative amylase sequences might occur between Ala_{15} and Gln_{16} in both the mouse salivary and pancreatic amylases. Cleavage there could result in the spontaneous cyclization of the N-terminal Gln residues to pyroglutamic acid (<Glu) residues. The N-terminal tryptic peptides of the secreted amylases would then be expected to have the following sequences and net charges at pH 6.5:

Salivary amylase

16 20 25
<Glu–Tyr-Asp–Pro–His–Thr–Gln–Tyr–Gly–Arg (½–)

Pancreatic amylase

16 20 25
<Glu–Tyr–Asp–Pro–His–Thr–Ser–Asp–Gly–Arg (1½–)

where the substitution of Asp_{23} for Tyr_{23} in pancreatic amylase confers on it a greater net negative charge. That difference would also be expected to make the pancreatic peptide less hydrophobic and thus it should elute earlier from a gel filtration column.

Figures 1 and 2 show elution profiles of Sephadex G-50 gel filtration chromatography of salivary and pancreatic amylase tryptic digests, respectively, and analytical peptide maps of the fractions. Tryptic digestion was incomplete in the case of salivary amylase as evidenced by the large protein core which elutes in the void volume peak of the column. None-the-less, comparable absorbance profiles and peptide maps were obtained for the two digestions although there was less peptide material in each peak of the salivary amylase profile. The peptide maps of the two amylases each contained a single His/Tyr positive spot which was not detectable with either cadmium–ninhydrin or fluorescamine (fig.1,2, →). Those peptides appeared late in elution profiles and had electrophoretic mobilities and staining characteristics consistent with the predictions.

The presumptive N-terminal tryptic peptides of the 2 amylases were purified from pools of fractions from their respective column peaks (fig.1,2, ——). The salivary amylase peptide eluted from the G-50 column in a peak (fig.1E) contaminated with salt. The pool of that peak was further fractionated by high-voltage paper electrophoresis at pH 6.5 and a large His/Tyr

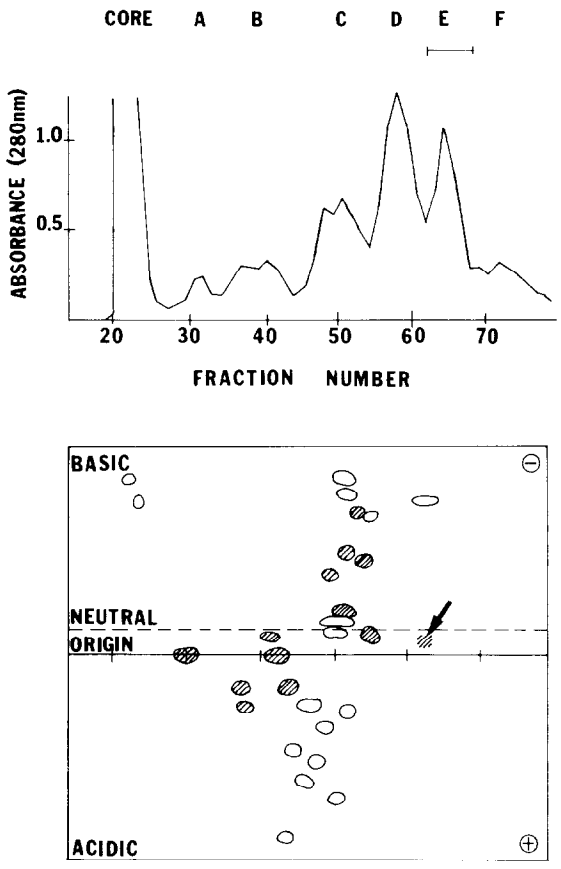

Fig.1. Sephadex G-50 gel filtration chromatography of peptides from mouse salivary amylase obtained by digestion with trypsin. The column was eluted with 0.1 M NH_4HCO_3. Column fractions were analyzed by high-voltage paper electrophoresis at pH 6.5 and the peptide map was stained with fluorescamine (outlined spots) and counterstained for His/Tyr (diagonal line shading). A spot which was His/Tyr positive but did not stain with fluorescamine (→) eluted in peak E.

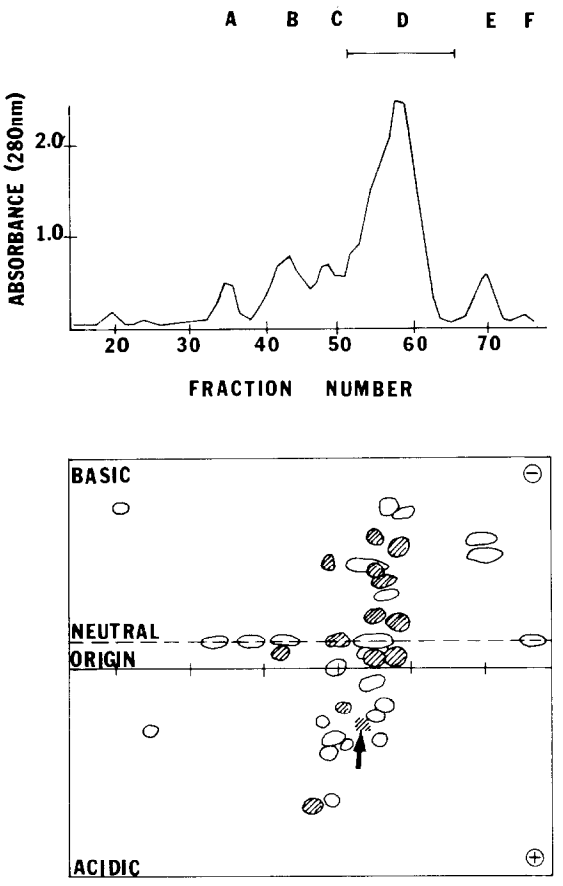

Fig.2. Sephadex G-50 gel filtration chromatography of peptides from mouse pancreatic amylase obtained by digestion with trypsin. The column was eluted and fractions analyzed as in fig.1. A spot which was His/Tyr positive but did not stain with fluorescamine (→) eluted in peak D.

staining area which ran slightly cathodal and included the origin was removed from the preparative map. The material eluted from that map was then subjected to high-voltage paper electrophoresis at pH 2.1 and a region staining for His/Tyr but not with cadmium–ninhydrin was cut from the paper and eluted. Analytical electrophoresis of small samples of the eluent yielded only a single peptide which stained for His/Tyr but was not detectable with either cadmium–ninhydrin or fluorescamine.

The pancreatic amylase peptide eluted from the G-50 column in an earlier peak (fig.2D) than had been the case for salivary amylase. That peptide pool was further fractionated by ion-exchange chromatography on DEAE-Sephacel (Pharmacia) with an NH_4HCO_3 gradient, followed by high-voltage paper electrophoresis at pH 6.5. A strip with strong His/Tyr staining but no detectable cadmium–ninhydrin staining was cut from the acidic side of the origin and eluted.

The blocked peptide obtained by trypsin digestion of salivary amylase was subdigested with chymotrypsin and two peptides obtained. One of those had a blocked N-terminus and an amino acid composition (Asx 1.2, Thr 0.8; Glx 2.1; Pro 1.0; Tyr 1.9; His 1.0) consistent with the first 8 residues of the anticipated N-terminal peptide. The other contained Gly (1.5) and Arg (1.0), with Gly as the N-terminus. Those

Table 1
Amino acid compositions of the N-terminal tryptic peptides of mouse salivary and pancreatic amylase

Amino acid residue	Salivary amylase		Pancreatic amylase	
	Obs.	Exp.[a]	Obs.	Exp.[a]
Asx	1.0	1	1.9	2
Ser	0	0	0.9	1
Thr	0.7	1	0.9	1
Glx	2.1	2	1.1	1
Pro	0.9	1	1.3	1
Gly	1.0	1	1.0	1
Tyr	1.8	2	1.0	1
His	0.9	1	1.0	1
Arg	1.2	1	1.1	1

[a] Predicted by the putative amino acid sequence derived from cDNA data [2], assuming cleavage of the signal peptide between Ala_{15} and Gln_{16}

findings are consistent with a 10-residue N-terminal peptide, ending in Gly–Arg, produced by a trypsin cleavage between Arg_{25} and Thr_{26}.

Elastase subdigestion of the corresponding blocked peptide from the trypsin digestion of pancreatic amylase yielded 3 peptides. One of those had a blocked N-terminus and an amino acid composition (Asx 1.0; Glx 1.0; Pro 1.0; Tyr 0.9; His 1.0) consistent with the first 5 residues of the anticipated N-terminal peptide. The other 2 had the amino acid compositions Thr (1.0), Ser (0.5) (Thr N-terminus) and Asx (1.0), Gly (1.1), Arg (1.0) (Asx N-terminus). Those findings are consistent with a 10-residue N-terminal peptide, ending in Thr–Ser Asp–Gly–Arg, produced by a trypsin cleavage between Arg_{25} and Thr_{26}.

The blocked peptides of the two amylases (residues 16–23 for salivary and residues 16–20 for pancreatic, respectively) produced by subdigestion of the tryptic peptides were further characterized by mass spectrometric analysis. The spectra obtained after deuteroacetylation and permethylation of 30 nmol salivary and 150 nmol pancreatic amylases confirmed the expected sequence: <Glu–Tyr–Asp– for the first 3 residues of each from signals at m/z 98, 317 and 460. Additional signals at m/z 70, 161 and 191 are in agreement with proline and tyrosine in the sequence. No peaks originating from sequence ions higher than 460 were found and no signals corresponding to naturally acetylated nor deuteroacetylated N-terminal Glu or Gln were present.

The occurrence of Ala_{15} in the amino acid sequence predicted for each amylase from its mRNA [2] makes it unlikely that trypsin digestion resulted in the blocked peptides we have studied since trypsin generally cleaves peptide bonds C-terminal to Lys and Arg residues. Therefore the <Glu residues identified by mass spectrometry must represent the true N-termini of the mouse amylases. In preliminary studies of the two mouse amylases, the carboxymethylated proteins were subjected to calf liver pyroglutamic amino peptidase (Boehringer) digestion [12] and then to Edman degradation [13]. High-performance liquid chromatography [14] detected PTH-tyrosine in those preparations but with low yield (~1%). It may be that the Tyr_{17} lowers the efficiency of pyroglutamic amino peptidase-catalyzed removal of the <Glu (originally Gln_{16}) N-termini of the 2 mouse amylases.

In contrast to these results, two groups have reported detecting an acetyl group associated with hog pancreatic amylase and suggested that it blocked the N-terminus [15,16]. We cannot rule out an acetylated N-terminus in the case of the hog enzyme, but it seems likely that the detection of acetyl groups in those studies can be explained by acetylation of the protein at a site(s) other than the N-terminus and/or minor contaminants containing acetyl groups.

A putative protein sequence for rat pancreatic amylase has also been deduced from cDNA data [17]. While no N-terminal peptide data has yet been reported for rat amylase, it seems likely that a similar cleavage site could be expected at Ala_{10} in the sequence [17]. Based on that prediction, the rat pancreatic amylase N-terminal tryptic peptide would be the same as that found in mouse pancreatic amylase, with the exception of an Ala substituted for a Ser.

Acknowledgements

This study was supported by funds from the Danish Natural Science Research Council (511-15512). R. C. K. was supported by USPHS Career Development Award 1 KO4 AMOO284, supplemented by funds from the University of Aarhus, and received travel support from the Danish Natural Science Research Council (511-20565). This is publication 80-34 from the Department of Medical Genetics, Indiana University School of Medicine. The authors express their appreciation for the technical assistance of Ms Margaret Clark, Ms Pia Ellegaard and Ms Gitte Rasmussen in the purification of the amylases and for the artwork of Mr Arno Jensen in the preparation of the figures.

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