TWO DISTINCT PANCREATIC AMYLASE GENES ARE ACTIVE IN YBR MICE

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ABSTRACT

The genetic determinants of pancreatic amylase expression in YBR mice differ in two respects from those of other inbred strains. First, there are two nonallelic amylase isozymes present in YBR pancreas, while most mouse strains express a single pancreatic amylase protein. In addition, the *in vivo* rate of total pancreatic amylase synthesis is 50% of that in other strains. Both these traits are determined by genetic sites in the region of the *Amy-2* locus on mouse chromosome *3*. To determine the molecular basis for the presence of two isozymes in this strain, we have compared portions of their amino acid sequences. Two differences between isozymes A_1 and B_1 were identified among the *77* residues compared. This result demonstrates that two distinct amylase genes are expressed in YBR pancreas.

THE amylase gene region on mouse chromosome *3* includes two closely linked loci, *Amy-1* and *Amy-2* (SICK and NIELSEN 1964; EICHER and LANE 1980; BLOOR and MEISLER 1980) *Amy-1* is expressed in parotid gland and in liver, while *Amy-2* is active in pancreas (HAGENBUCHLE, BOVEY and YOUNG 1980; YOUNG, HAGENBUCHLE and SCHIBLER 1981). The nucleotide sequence of portions of this gene region have recently been described (HAGENBUCHLE, BOVEY and YOUNG 1980; HAGENBUCHLE *et al.* 1981; YOUNG, HAGENBUCHLE and SCHIBLER 1981; SCHIBLER *et al.* 1980). Genetic variation in the numbers of amylase proteins and their rates of synthesis in parotid gland and pancreas have been described in inbred and feral mouse populations (BLOOR, MEISLER and NIELSEN 1981; HJORTH, LUSIS and NIELSEN 1980; HJORTH 1979; KAPLAN, CHAPMAN and RUDDLE 1973; NIELSEN and SICK 1975). The availability of molecular probes and the existence of genetic variation in gene expression make the mouse amylase region an attractive model for studies of mammalian gene regulation.

The pancreatic amylase phenotype of strain YBR is of particular interest. YBR pancreas contains two nonallelic amylase isozymes, designated A_1 and B_1, rather than the single A_2 isozyme present in the pancreas of most other strains (HJORTH, LUSIS and NIELSEN 1980; BLOOR, MEISLER and NIELSEN 1981). The A_1 and B_1 isozymes differ in their electrophoretic mobility, rates of synthesis, and isoelectric points, which are pH 7.3 and pH 7.8 respectively (STRAHLER, HEWITT-EMMETT and MEISLER 1981). Genetic studies have demonstrated the presence

of *cis*-acting sites in the amylase gene region that determine the quantitative expression of each isozyme (BLOOR, MEISLER and NIELSEN 1981). The genetic and biochemical data can be explained by the hypothesis that two distinct, nonallelic structural genes are active in YBR pancreas, one encoding each isozyme. However, alternative explanations based on differential processing of a single gene product into two isozymes have not been excluded. To provide definitive evidence regarding the molecular differences between the A_1 and B_1 isozymes, we undertook a study of their amino acid sequences.

MATERIALS AND METHODS

Animals. Inbred YBR/Ki mice were purchased from Kirschbaum Memorial Laboratories, Kent, Ohio. The congenic strain C3H.Amy^{YBR}, carrying the amylase gene region from strain YBR on a C3H/As genetic background, was described in an earlier publication (BLOOR, MEISLER and NIELSEN 1981). Since there were no differences in the IEF patterns of cyanogen bromide peptides for these two strains (see below), amylase from both sources was combined for the sequencing studies.

Purification of amylase isozymes A_1 and B_1. Pancreas was homogenized in 0.05 M Tris HCl, pH 7.5 with a Poilytron homogenizer (BRINKMANN). The homogenate was centrifuged at 12,000 $\times$ g_{max} for 20 min. The pH of the supernatant solution was adjusted to pH 5.5 with acetic acid; after 15 min at 0° the precipitated material was removed by recentrifugation. Amylase was then purified by affinity chromatography on cycloheptaamylose (HJORTH, LUSIS and NIELSEN 1980; SILVANOVICH and HILL 1976). The purified amylase was free from contaminating proteins as judged by electrophoresis in the presence of SDS. Amylase activity was assayed as previously described (BLOOR, MEISLER and NIELSEN 1981); protein was determined by the Lowry method. The purified amylase protein was applied to a horizontal granular bed of Sephadex G75 (6g) containing 3.2% ampholytes (LKB, pH 6–9.5), in an LKB Multiphor apparatus. After focusing at 8 watts constant power (1200 volts maximum) for 19–24 hr at 9°, fractions were removed and the enzyme was eluted from the Sephadex with water. The activity and pH of each fraction was measured. Recovery of amylase activity was greater than 95% for the preparative isoelectric focusing step. After appropriate fractions were pooled, the affinity chromatography step was repeated to remove ampholytes and concentrate the amylase. Purified amylase was stored at —20°.

Cyanogen bromide cleavage: The purified isozymes, 1–2 mg/ml, were dialyzed against 70% formic acid and then incubated with cyanogen bromide in a 200-fold molar excess over methionine for 20 hr at room temperature in the dark. The reaction mixture was diluted 20-fold with water and lyophilized.

Preparative isolation of cyanogen bromide fragments: Cyanogen bromide fragments were isolated by preparative isoelectric focusing in an LKB electrofocusing column (110 ml) with a sucrose gradient (6–42%) containing 1% ampholyte (pH 4 to 7), 6 M urea and 0.01 M dithiothreitol. Focusing was carried out at 15° for 24 hr at 10 w constant power (1600 v maximum) with the cathode as the lower reservoir. Since a sharp band of precipitated material formed at the interface of the pH gradient and the cathode reservoir, the first 30 ml collected from the gradient were discarded. Fifty 1.4 ml fractions were then collected and their absorbance at 280 nm was measured. Fractions containing the major acidic fragment were pooled and desalted on a BioGel P2 column (2.5 cm $\times$ 70 cm) equilibrated with 0.5% formic acid.

Preparation and separation of tryptic peptides: The desalted cyanogen bromide fragments were lyophilized and then dissolved in 0.2 M ammonium bicarbonate buffer, pH 8.2, at a concentration of 1 mg/ml, and incubated with TPCK-trypsin (Worthington), 10 μg/ml, for 4 hr at 37°. An additional 10 μg/ml of trypsin was added to the reaction after the first two hours. The digest (10 nmole) was lyophilized and dissolved in 0.1% trifluoroacetic acid for reverse phase HPLC separation using a Beckman Model 332 HPLC System. Peptides were separated on

a 0.4 × 30 cm μBondapak C_{18} column (Waters Associates) with a 0.4 × 10 cm guard column (CO:Pell, ODS, Whatman). Solvents contained trifluoroacetic acid (BENNETT *et al.* 1979; HENDERSON, SOWDER and OROSZLAN 1981; MAHONEY and HERMODSON 1980). Solvent A was 0.1% trifluoroacetic acid in HPLC grade water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. The flow rate was 1 ml/min. Absorbance was monitored at 215 nm with a Hitachi variable wavelength spectrophotometer.

Sequence analysis of tryptic peptides: Peptides were isolated by reverse phase HPLC as described above except that 100 to 150 nmoles of tryptic digest were injected. Peak fractions were collected manually. Sequencing was carried out on a Beckman 890B Sequenator (HENRIKSSON, TANIS and TASHIAN 1980). One mg of human carbonic anhydrase I, whose N-terminus is blocked, was added to the reaction cup to promote retention of the sample (HENRIKSSON, TANIS and TASHIAN 1980). PTH-amino acids were identified by HPLC as described by KAGEOKA *et al.* (1981), except that an Altex Ultrasphere-ODS column (5 μ, 0.46 × 25 cm) was used. All PTH-amino acids could be identified by this method with three exceptions: PTH-Arg and PTH-His, which remain in the aqueous phase after conversion, and PTH-Trp, which is obscured by artifact peaks due to the DMAA sequencing buffer. The superposition of PTH-Met and PTH-Val was not a problem because the cyanogen bromide cleavage eliminates Met residues. PTH-Lys and PTH-Ile were sufficiently resolved to permit unambiguous identification. PTH-Arg and PTH-His were identified by staining with phenanthrenequinone and Pauly reagent respectively. PTH-Trp was identified by thin layer chromatography (WALZ and REUTERBY 1975). Asparagine and glutamine residues were always accompanied by some of the free acid; the relative peak height of the amide was taken into account for the assignment of these amino acids. This was particularly critical in the sequences Asp-Asn-Asn (positions 17–19) and Asn-Asn-Asp-Asp (positions 36–39).

Identification of tryptic peptides: Tryptic peptides were identified by comparison of their sequence with that predicted by the cDNA sequence for mouse pancreatic amylase (HAGENBUCHE, BOVEY and YOUNG 1980). All the expected tryptic peptides were identified. Peptide T2 was sequenced as a mixture of T2 and T2′. The amino acid composition and partial sequence of T2′ suggested that this peptide was derived from T2 by nonspecific cleavage at Phe 25 (data not shown). This interpretation is supported by the fact that the mixture of T2 and T2′ gave a single amino acid sequence, and the observation that the expected dipeptide product of such a cleavage, Ser-Arg, (positions 26 and 27), was identified in the unretained HPLC fraction (3 min). With regard to the two minor peaks, the material which is eluted 0.4 min before T2 is identical in amino acid composition to T2, and the amino acid composition of the component eluting 0.7 min before T2′ is identical to that of T2′. The component designated T4-T5 had an amino acid composition consistent with the sum of peptides T4 plus T5, including one mole each of lysine and arginine and two moles of cysteine. A single sequence was obtained from the material in this region of the chromatogram. We conclude that the peak labeled T4–T5 comprises residues 31–72, and that the tryptic cleavage at Lys 63 did not occur, presumably because of the adjacent Asp 62. The only additional tryptic cleavage product predicted by the cDNA sequence is the putative C-terminal leucine, which would not be detected on this chromatogram. The peak at 24.3 min was observed when trypsin alone was autodigested and analyzed. The first 12 residues of the peptide which is eluted at 28.8 min is identical to the sequence of T7. The material which is eluted at 44.1 min disappears after 8 hr of tryptic digestion; no additional peaks are generated during this period, although the ratio of T2 to T2′ decreases significantly.

RESULTS

To compare the sequence of the two amylase isozymes, the purified proteins were isolated. Total pancreatic amylase from C3H.Amy^{YBR} or YBR/Ki was prepared by affinity chromatography on cycloheptaamylose as described in MATERIALS AND METHODS. The A_1 and B_1 isozymes were then separated by preparative isoelectric focusing (Figure 1). Electrophoresis at pH 8.1 (BLOOR, MEISLER

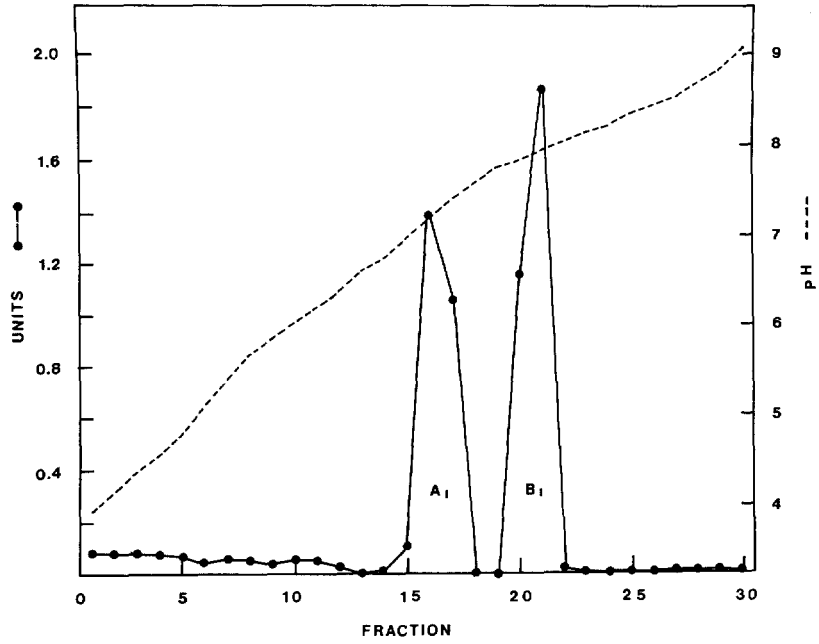

FIGURE 1.—Separation of pancreatic amylase isozymes A_1 and B_1. Purified pancreatic amylase (0.7 mg) from strain YBR was fractionated by isoelectric focusing on Sephadex G75, as described in MATERIALS AND METHODS. The amylase activity of 50 μl aliquots from each fraction was determined.

and NIELSEN 1981) was used to verify the identity of each isozyme. The specific activities of isozymes A_1 and B_1 did not differ significantly, and were 2160 ± 200 and 2350 ± 80 units/mg, respectively (mean ± SD for four preparations). A small difference in thermostability of the isozymes was observed when the rate of inactivation at 46.5° was compared (Figure 2); the B_1 isozymes is the more stable, with a halflife of 42 min, compared with 23 min for A_1. This observation is further evidence of a structural difference between the isozymes.

To identify possible structural differences, the isozymes were cleaved with cyanogen bromide, and the resulting fragments were analyzed by isoelectric focusing (Figure 3). The overall patterns of fragments from the two isozymes are quite similar. However, the isoelectric point of the major acidic fragment from the A_1 isozyme is 0.3 pH units lower than that of the corresponding fragment from isozyme B_1.

We isolated this cyanogen bromide fragment from each isozyme by preparative isoelectric focusing (Figure 4). Amino acid analysis indicated that the fragment from B_1 contained approximately two moles more alanine and one mole less glutamic acid than the fragment from A_1. Identification of the cyanogen bromide fragment and its tryptic peptides was possible by comparison of its sequence with the recently reported cDNA sequence for pancreatic amylase A_2 from strain A/J (HAGENBUCHLE, BOVEY and YOUNG 1980). The first 28 residues of the

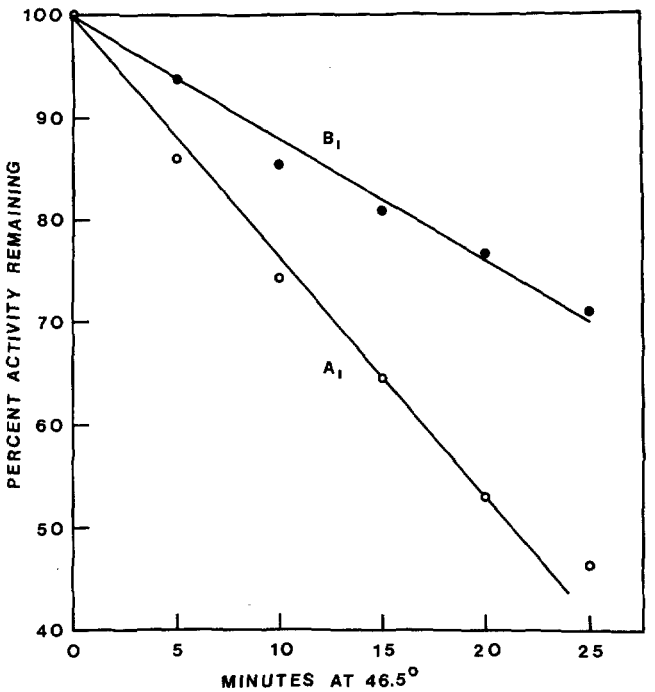

FIGURE 2.—Thermal denaturation of amylase isozymes. Purified enzyme ($\simeq$ 0.02 mg/ml) was incubated at 46.5° for the indicated time in 0.044 M sodium phosphate buffer, pH 6.9 containing bovine serum albumin, 0.1 mg/ml. The residual activity was assayed under standard conditions.

pI = 5.2 fragment from isozyme A_1 were sequenced; the results agreed with the sequence predicted for the 102-residue C-terminal cyanogen bromide fragment of amylase A_2. Additional sequencing was carried out on tryptic peptides isolated from this major acidic cyanogen bromide fragment by reverse phase HPLC (Figure 5). Each peptide was identified by comparison of its amino acid sequence with that predicted by the cDNA sequence. All tryptic peptides predicted from the cDNA sequence were identified; they are numbered sequentially from the N-terminus of the cyanogen bromide fragment. The peptide designated T2′ is a cleavage product of peptide T2; the peptide designated T4–T5 is a single peptide resulting from the lack of cleavage between the expected peptides T4 and T5 (discussed in MATERIALS AND METHODS).

The HPLC elution profiles of tryptic peptides from isozymes A_1 and B_1 were then compared. Peptides T1, T3, T6 and T7 from the two isozymes had identical retention times. Differences were observed in the elution time of peptide T2 and its derivative T2′, and also of peptide T4–T5 (Figure 6). When these peptides were sequenced, one amino acid substitution in peptide T2 and one in peptide T4–T5 were identified (Figure 7). At position 13 of peptide T2, there is a serine residue in amylase A_1 and an alanine residue in B_1. At position 44 in pep-

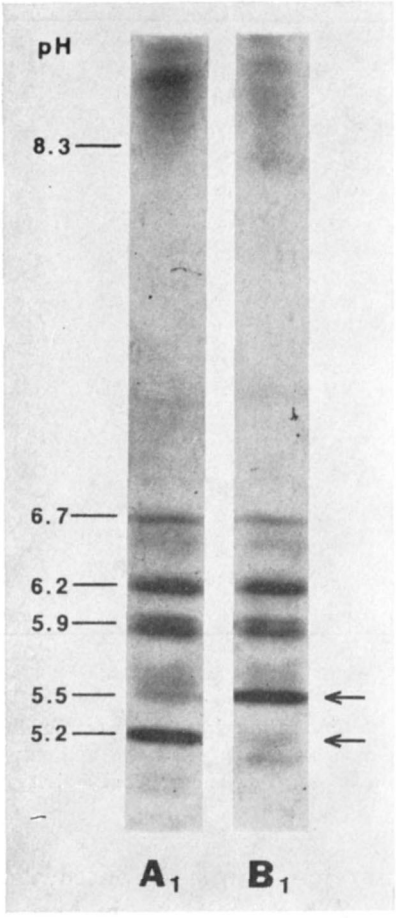

FIGURE 3.—Analytical isoelectric focusing of cyanogen bromide fragments. Purified amylases A_1 and B_1 were treated with cyanogen bromide as described in MATERIALS AND METHODS. A 50-μg aliquot was then dissolved in 30 μl of 2% ampholyte solution (pH 3.5–10) (LKB) containing 6 M urea and 0.01 M dithiothreitol, incubated for 30 min at room temperature, and applied to a 6% polyacrylamide gel containing 2% ampholytes (pH 3.5–10) and 6 M urea. Isoelectric focusing was carried out at 13° for 3 hr at 10 watts constant power (1200 volts maximum). After incubation in 50% trichloroacetic acid overnight to remove ampholytes, the gel was stained with Coomassie Blue R250. The pH gradient was measured on a gel slice removed immediately after focusing. The major acidic cyanogen bromide fragments from amylases A_1 and B_1 have isoelectric points of pH 5.2 and pH 5.5, respectively (arrows).

tide T4, there is a glutamic acid residue in A_1 and an alanine residue in B_1. In both cases, the greater hydrophobicity of the residues in the B_1 peptides can account for the longer retention time on the reverse phase HPLC column (MEEK 1980). In addition, the extra acidic residue in A_1 can account for the lower isoelectric point of the intact A_1 isozyme (Figure 1) and of its cyanogen bromide fragment (Figure 3). These two differences between the amino acid sequence of nonallelic isozymes A_1 and B_1 demonstrate that there has been duplication of at least the C-terminal portions of the *Amy-2* structural gene.

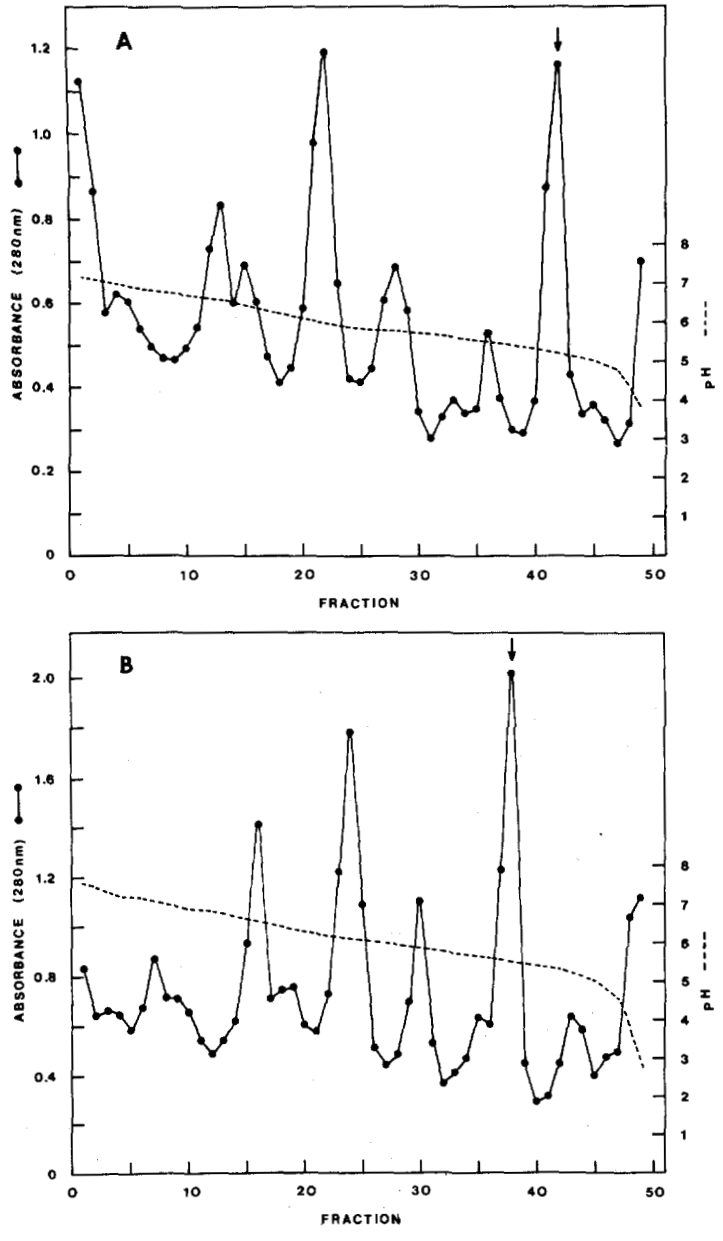

FIGURE 4.—Preparative isoelectric focusing of cyanogen bromide fragments. Cyanogen bromide fragments were separated by isoelectric focusing in a preparative column as described in MATERIALS AND METHODS. A. Cyanogen bromide fragments from 9.7 mg of amylase A_1. B. Cyanogen bromide fragments from 12.3 mg of amylase B_1. Fractions containing the major acidic fragments with pI = 5.2 or 5.5 (arrows) were pooled for further analysis.

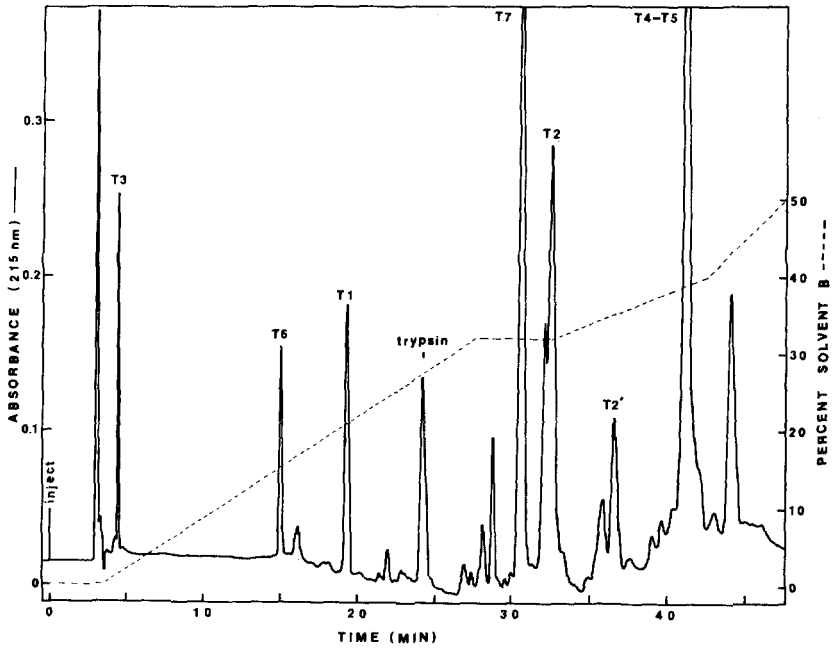

FIGURE 5.—Reverse phase HPLC of tryptic peptides. The cyanogen bromide fragment (pI=5.2) from amylase A1 was digested with trypsin as described in MATERIALS AND METHODS. Tryptic peptides were separated by HPLC on a μBondapak C_{18} column. Each peptide was identified by comparison of its amino acid composition and sequence with the cDNA sequence for pancreatic amylase (see text). Peptides are numbered T1 to T7, starting from the amino-terminus of the cyanogen bromide fragment. The peptide designated T2′ is a cleavage product of peptide T2; the peptide designated T4–T5 is a single peptide resulting from the lack of cleavage between the expected peptides T4 and T5 (see MATERIALS AND METHODS).

DISCUSSION

Amylases A_1 and B_1 differ at two of the 77 amino acid residues that we have compared (Figure 7). In the common pancreatic amylase isozyme, A_2, we have identified residues Ser 13 and Ala 44 in this cyanogen bromide fragment (STRAHLER, HEWETT-EMMETT and MEISLER 1981; and unpublished observations on A_2 from strain C3H). Thus, a single base substitution can account for the conversion of the A_2 isozyme to A_1 or to B_1, but two base substitutions are required to interconvert A_1 and B_1. This suggests that duplication of an A_2-like sequence preceded the divergence of A_1 and B_1. If this is correct, we may expect to find two *Amy-2* genes in some inbred strains with the A_2 phenotype. In agreement with this expectation, recent analysis of genomic DNA from A/J mice by quantitative autoradiography of restriction fragments indicates that there are two copies of the *Amy-2* gene in this strain (SCHIBLER *et al.* 1982).

The Ala 13 residue in B_1 and the Glu 44 in A_1 are identical to the corresponding residues of the salivary amylase protein (HAGENBUCHLE, BOVEY and YOUNG 1980). However, both A_1 and B_1 differ from the salivary amylase sequence at

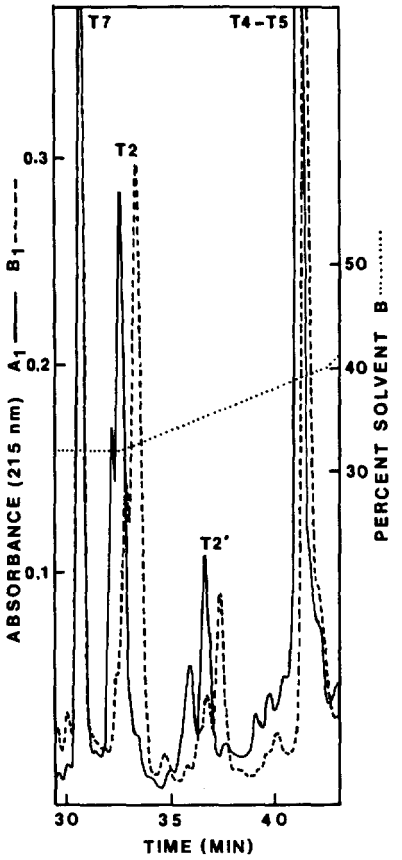

FIGURE 6.—Comparison of the HPLC elution profiles of tryptic peptides from isozymes A_1 and B_1. HPLC was carried out as described in the legend to Figure 5. The results of two representative chromatographs are presented. Peptides T2 and T2′ from B_1 were eluted 0.8 min later than the corresponding peptides from A_1. Peptide T4–T5 from B_1 was eluted 0.4 min later than the corresponding peptide from A_1. When mixtures of peptides from A_1 and B_1 were analyzed, T2, T2′ and T4–T5 appeared as split peaks. Solid line, A_1; dashed line, B_1.

seven other residues in this region. They are thus more closely related to each other than to the product of the *Amy-1* locus. The identity of the substituted residues in A_1 and B_1 with the residues in salivary amylase appears to be the result of "convergent" evolution, possibly reflecting the compatibility of these particular alternative residues with the tertiary structure of the active enzyme. Residues 13 and 44 are also among the sites of differences between rat pancreatic amylase and mouse pancreatic amylase A_2 (MACDONALD *et al.* 1980).

HJORTH, LUSIS and NIELSEN (1980) compared the cyanogen bromide fragments of pancreatic amylase isolated from various mouse strains. They found two acidic fragments in pancreatic amylase from strain YBR, which they designated F and S; these correspond to the fragments with isoelectric points 5.2 and 5.5 in Figure 3. The F and S fragments can now be assigned to the A_1 and B_1

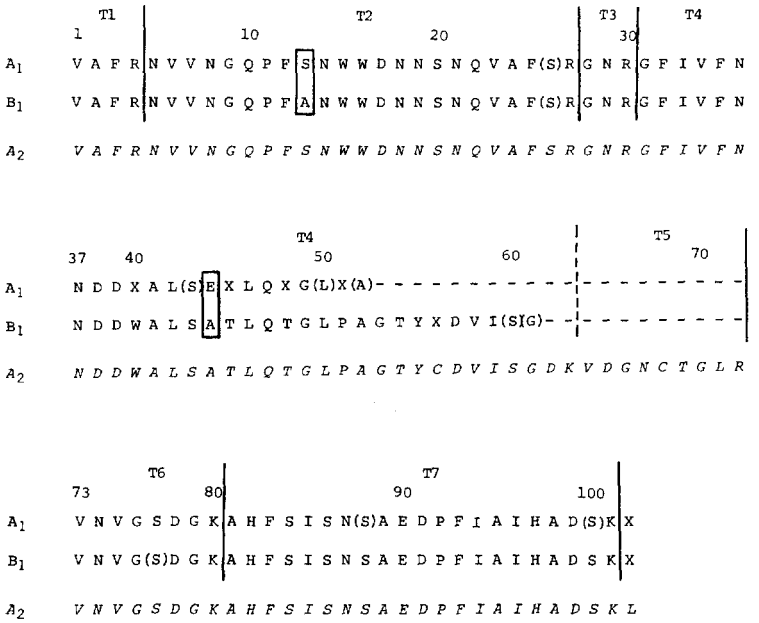

FIGURE 7.—Partial sequence of the acidic cyanogen fragment from pancreatic amylases A_1 and B_1. Tryptic peptides T1, T2, T3, T6 and T7 were completely sequenced; T4–T5 was partially sequenced. In addition, the intact cyanogen bromide fragments from A_1 (residues 1–28) and B_1 (residues 1–32) were sequenced. The italicized sequence for amylase A_2 is that predicted by the cDNA sequence (HAGENBUCHLE, BOVEY and YOUNG 1980). Amino acid residue 1 in this figure is encoded by nucleotides 1321–1323 in the published cDNA sequence (HAGENBUCHLE, BOVEY and YOUNG 1980). Tryptic cleavage sites are indicated by solid lines. The two amino acid differences between isozymes A_1 and B_1 are boxed.

isozymes respectively. The more acidic (F) fragment was detected by HJORTH and coworkers in all strains with an A_1 pancreatic isozyme, suggesting a common structure for the A_1 isozymes in various strains.

The activity of multiple amylase genes has previously been postulated in order to account for the presence of multiple pancreatic isozymes in characteristic ratios in various mouse stocks (HJORTH, LUSIS and NIELSEN 1980; SICK and NIELSEN 1964; NIELSEN and SICK 1975). The differences in primary sequence of YBR isozymes A_1 and B_1 reported here provide direct evidence for this model. Our results demonstrate that a minimum of two genes are active in pancreas of YBR mice. The basis for the unequal rates of synthesis of the A_1 and B_1 proteins in YBR pancreas (BLOOR, MEISLER and NIELSEN 1981) may reside in the structure of the two genes. Multiple amylase-related sequences have been detected by hybridization techniques in genomic DNA from mouse (YOUNG, HAGENBUCHLE and SCHIBLER 1981; OWERBACH, NIELSEN and RUTTER 1981) and rat (MACDONALD *et al.* 1980), but it is not known how many of these are active. Further investigation of the structural organization of the amylase genes in

strain YBR should contribute to our understanding of the regulation of this multi-gene family.

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