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Hepatic α-amylase in rat

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α-Amylase activity and malto-oligosaccharides have long been described without isolation of any relevant enzyme in mammalian liver. We developed a micro-assay method for malto-oligosaccharides and effectively applied it to the study of hepatic amylolysis and isolation of an α-amylase from rat liver. The liver showed high α-amylase activity, high enough to account for the whole glycogenolytic activity in the liver. The isolated enzyme was digested with lysylendopeptidase and the major peptide fragments were sequenced. Complete amino acid sequencing was based on the full coding sequence of its mRNA determined by RT-PCR (Accession number: AB057450). The hepatic neutral amylolytic activity is so high that we expect the amylolytic pathway to be significant in glycogen metabolism in the liver.

Key words: rat, liver, alpha-amylase, cDNA sequence, oligosaccharide, O-ethylhydroxylamine.

Introduction

We have demonstrated that a leukemic cell line and rat liver produce a unique anhydrohexulose, 1,5-anhydrofructose (1,5AnFru) (KAMETANI et al., 1996; SUZUKI et al., 1996). The anhydrohexulose is present most abundantly in the liver (KAMETANI et al. 1996), the organ which plays the central role in supplying glucose to maintain glycemic levels in the post-absorptive period. Other organs contain much less 1,5AnFru while the plasma contains practically none. We have also demonstrated that a partially-purified α-1,4-glucan lyase produces 1,5AnFru from malto-oligosaccharides rather than glycogen (HIRANO et al., 2000). Those results indicate that the third glycogenolytic pathway to 1,5AnFru shares an amylolytic step with the hydrolytic pathway to glucose, in marked contrast to fungal and algal 1,5AnFru production (YU & PEDERSÉN, 1993; YU et al., 1993, 1995, 1999). Hepatic α-amylases and oligosaccharides have occasionally been reported for more than fifty years without isolation of the enzyme (MACGEACHIN & POTTER, 1960; BROSEMER & RUTTER, 1961; RUTTER & BROSEMER, 1961; RUTTER et al., 1961; MORDOH et al., 1968). Here we effectively applied an oxime-derivatization method to oligosaccharide assay in the liver and reproduced the old observations that rat liver contained very large amounts of α-amylase and appreciable amounts of malto-oligosaccharides.

Material and methods

DE 52 was purchased from Whatman International

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(Kent, England), Fast Flow Q- and SP- Sepharoses, Mono Q, Superose 12 and Sephacryl S-300, from Pharmacia Biotech Inc. (Uppsala, Sweden), a short (4.6 × 150 mm) and a long (4.6 × 250 mm) reverse phase column, Kromasil KR100-5C18s were the products of EKA chemicals, and α-glucosidase from *Saccharomyces* sp., *O*−Ethylhydroxylamine/HCl, HEPES (2[4-(2-hydroxyethyl)-1-peperazinyl]ethansulfonic acid), Tricine (N-[tris(hydroxymethyl)methyl]-glycine), Tris (2-amino-2hydroxymethyl-1,3-propanediol) and all other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Amylase and oligosaccharide extractions from rat liver
After several days of preliminary feeding, four male Wistar rats (nine weeks of age) with body weights of 250–280 g were sacrificed under anaesthesia with ether by depletion of the whole blood from the radial artery and descending aorta. The liver was removed from the depleted animals and either immediately dipped and frozen in liquid nitrogen (for RNA preparation) or quickly rinsed with 150 mM NaCl and then frozen for later use. On the day of extraction, several pieces (ca. 400 mg in total) were excised from a frozen liver, weighed and placed in the glass cylinder of a homogenizer with Teflon pestle and homogenized with 1.6 mL of 250 mM sorbitol by 5 strokes. One-fifth portion of the homogenate was used for oligosaccharide assay. The remainder was acidified with exactly one hundredth volume of 1 M acetic acid to pH 5.0 and centrifuged at 20000 *g*. The resulting supernatant was neutralized to pH 8.0 and denoted as the acidic extract. The precipitate was thoroughly suspended in 1.6 mL of 10 mM Tricine buffer, pH 8.4 and again centrifuged at 20000 *g* to separate the supernatant, which was denoted as the first alkaline extract. The alkaline extraction from the precipitate was repeated to obtain the second, third, etc., alkaline extracts. A 10 μL portion each of these extracts and 50 μL water were added to 10 μL of 10 mg/mL glycogen in 100 mM phosphate buffer pH 7.0 containing 10 mM NaCl, and incubated at 37 ℃ for 30 min. The α-amylase reaction was terminated by the addition of four-times volume of ethanol, and glucose, maltose and oligosaccharides in the ethanolic suspension were assayed as described below.

Malto-oligosaccharide assay
The ethanolic suspension resulting from the above assay was centrifuged at 20,000 *g* for 5 min to obtain a clear oligosaccharide extract. The whole extract was then dried in a centrifugal evaporator and the residue was re-dissolved in 100 μL of 1.25% ethylhydroxylamine hydrochloride solution and heated at 80 ℃ for 30 min. The whole reaction mixture was applied to analysis of the oximes of glucose, maltose, and other malto-oligosaccharides on the shorter reverse phase column developed with an increasing acetonitrile gradient in water (0–12% of acetonitrile in 6 min). In the case of oligosaccharide assay in the original homogenate, a 100 μL portion was used. After deproteinization with four times volume of ethanol and evaporation, the resulting residue was reconstituted to a 100 μL aqueous solution containing 1.25% ethylhydroxylamine hydrochloride and the solution was heated at 80 ℃ for 30 min. A small portion was subjected to the oxime analysis on a reverse phase column. An automated HPLC (LC10, Shimadzu, Kyoto) was used for these analyses and the elution was monitored by UV absorption at 210 nm.

Purification of rat hepatic α-amylase and amino acid sequencing
Purification of rat hepatic α-amylase will be described in detail elsewhere. Briefly, the amylase extraction described above was adapted to a larger scale; the acid extract and the 1st to fourth alkaline extracts from 100g rat livers were applied to a DE52 column (50 mL in HEPES form) equilibrated with 10 mM HEPES buffer pH 7.4 (HEPES buffer A). After the column was washed with 3-column-volumes of the same buffer, the amylase was eluted with 10 mM HEPES buffer containing 150 mM NaCl (HEPES buffer B). The active fraction was concentrated on an ultrafiltration membrane (Amicon YM10) to 10 mL, and was applied to a Sephacryl S-200 column (200 mL) equilibrated with 10 mM Tris-HCl buffer containing 50 mM NaCl and 0.1% Tween 20 (Tris buffer B). The column was then eluted with Tris buffer B. The first 0.8 column volume of the eluate was separated and all the succeeding eluate up to 4-column-volumes was collected and again concentrated on YM10 and thoroughly de-ionized on the same membrane by repeated cycles of dilution with 10 mM Tris-HCl buffer containing 0.1% Tween 20 (Tris buffer A) and concentration. The de-ionized concentrate was then applied to the second DE 52 column (50 mL) equilibrated with Tris buffer A and the amylase was eluted with a linear gradient of 0 to 300 mM NaCl in Tris buffer A. The crude α-amylase thus obtained was further purified to homogeneity by ion-exchange and gel permeation chromatography on Fast Flow Q-Sepharose, Superose 12 and MonoQ columns.

A portion of the purified α-amylase (ca. 50 mAbs/mL) was digested with 2 pmoles of lysylendopeptidase in 10 mM Tricine buffer (pH 8.4) containing 2M urea for 4 h. The resulting digest was directly applied to the longer reverse phase column and was eluted with a gradient of 0–30% acetonitrile in water. All the chromatographic peaks were separately collected in test tubes and each fraction was dried and subjected to amino acid sequencing.

Cloning and sequencing of cDNA for hepatic α-amylase
The cloning of the cDNA for hepatic α-amylase will be described in detail elsewhere. Briefly, total RNA was extracted from the frozen liver using TRIZOL reagent (GIBCO BRL) according to the instruction of the manufacturer. The single strand cDNA was then prepared by *in vitro* synthesis of cDNA with avian myeloblastosis virus (AMV) reverse transcriptase (supplied as a part of 5'/3'RACE kit from Boehringer Mannheim) using total RNA (5 μg) and the oligo dT-anchor primer also supplied in the kit. Synthesized primers such as

T_{18} A/G/C and several other primers directed to the possible internal sequences estimated from the known sequence of the mouse salivary enzyme were also used. A 25 cycle-amplification (94℃ for 30 sec, 52℃ for 30 sec and 72℃ for 50 sec) was applied to the single strand cDNAs using appropriate pairs of the primers. Nested PCR was then applied to those PCR-amplified cDNAs and the final products were isolated by electrophoresis on a low melting agarose gel and cloned into pCR2.1-TOPO vector (TOPO TA Cloning kit, Invitrogen). For sequencing, Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) was used.

The nucleotide sequence thus obtained was deposited in DDJB/Genbank/EMBL database under the accession number, AB057450.

Results and discussion

Validity of oligosaccharide assay method

The oxime method employed in this study was originally developed for 1,5AnFlu assay. In that application, it was demonstrated that the derivatization was efficient and reproducible even when applied to a trace amount (pico-mole order) of 1,5AnFlu in a crude sample (KAMETANI et al., 1996). Although glucose and maltosaccharides have less reducing activity than 1.5AnFlu, our preliminary study indicated that their oxime formation was still efficient and not so much influenced by the heavy contamination of proteins and other biomolecules ordinarily present in the liver homogenate. Fructose forms its oxime less efficiently and, for satisfactory derivation, the coupling reaction needs to be carried out at 100℃ (KAWASAKI et al., 2002). The *O*-ethyloxime derivatives of glucose, maltose and oligosaccharides showed most convenient retardation on the ODS column; glucose and maltose formed isolated peaks while maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose formed a fused cluster of chromatographic peaks with maltopentaose showing the longest retardation (Fig. 1). Since the relative elution times of these oligosaccharides are sensitively and differently influenced by the acetonitrile gradients, one may adjust it so as to obtain maximal overlaps of maltooligosaccharide peaks. In the present study, we separately determined glucose and maltose amounts from their peak heights and the sum of maltotriose through maltoheptaose amounts from the peak area of the fused peaks. The peak heights and areas were calibrated to respective molar amounts by comparing with those of the respective authentic compounds used as standards. For this calibration, we confirmed that the molar absorptions of oximes for maltotriose through maltoheptose were almost equal.

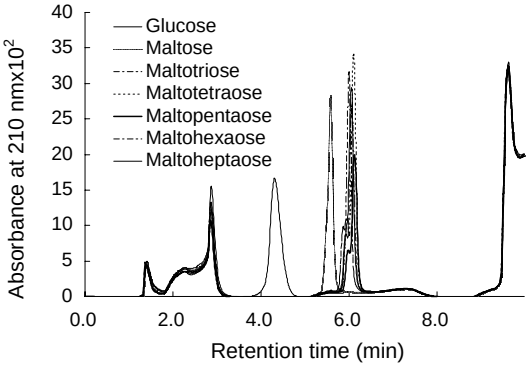

Fig. 1. Elutions of authentic ethyloxime derivatives of glucose, maltose and maltooligosaccharides. 50 nmole each of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, or maltohepataose were separately converted to the respective ethyloxime and subjected to HPLC analysis on a reverse phase column as described in the text. The order of peak elution was glucose (4.31 min), maltose (5.58 min), maltotriose (6.00 min), maltoheptaose (6.06 min), maltohexaose (6.07 min), maltotetraose (6.11 min) and maltopentaose (6.13 min).

Maltooligosaccharides and α-amylase in the liver

A large amount of sucrose gives rise to glucose ethyloxime and another yet unidentified derivative whose chromatographic peak overlaps with that for maltose ethyloxime on the reverse phase column. For this reason we used sorbitol for adjustment of osmosis in the liver homogenate. A variable concentration of glucose was observed in the alcoholic extract of the rat liver; the concentration was often much higher than the value expected from the glycemic level (Fig. 2). This can be due to post-mortem glucose production from glucose phosphates and/or glycogen. A small but significant amount of oligosaccharide was demonstrated to have been present by disappearance, after α-glucosidase treatment, of the major part of the small peak at the retention time corresponding to maltooligosaccharide (Fig. 2). The thorough α-glucosidase treatment concomitantly produced much more glucose than was expected from the disappearance of the maltooligosaccharide peak, thus indicating the presence of other α-glucosidase substrates larger than maltoheptaose. The glucose-equivalent of these oligosaccharides in the liver seemed to exceed the glucose concentration in the circulation.

More than fifty per cent of α-amylase activ-

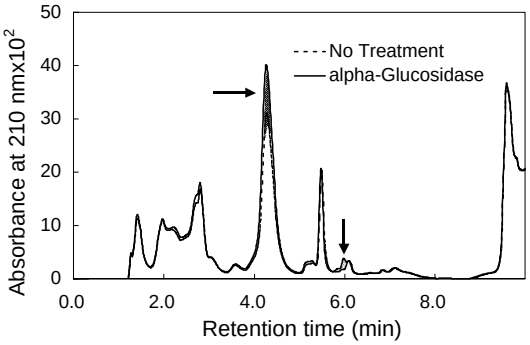

Fig. 2. Oligosaccharides in rat liver homogenate. A fixed amount of alcoholic liver extract (corresponing to 2.5 mg liver) was incubated with or without α-glucosidase (ca. 1 unit) for 30 min at 37℃ in 100 μL of 20 mM phosphate buffer pH 7.0. The reaction was terminated by addition of four volumes of ethanol and the resulting ethanolic extracts were treated and applied to a reverse phase column as described in the text. The shaded area indicated by the vertical arrow at 6 min indicates the peak area that disappeared upon α-glucosidase treatment, and the shaded area on the glucose peak marked with the horizontal arrow shows the production of glucose upon α-glucosidase treatment, which corresponds to a glucose increment of 11 μmol/g liver.

ity was taken into solution only after repeated extraction of the particulate fraction under mild alkaline conditions. In the present study, roughly half of the amylase activity in the particulate fraction was extracted into the solution in each alkaline extraction. Thus we estimate that twice the activity of the α-amylase in the first alkaline extract originally resides in the particulate fractions (Fig. 3). More than half of the solubilized α-amylase activity could be retained on an anion-exchange column (DE 52) while the remainder could be retained on a cation-exchange column (SP-Sepharose). Only the α-amylase retained on the anion column was subjected to further purification. The employment of HEPES as the counter ion of the anion exchanger was critical for the feasibility of the succeeding chromatographic purification; the α-amylase formed distinct chromatographic peaks on Sepharose-based and synthetic polymer-type columns after this treatment. We observed that the pH of the eluate containing α-amylase activity was raised a little over 9 due to the exchange of HEPES anions for chloride on the column.

Primary structure

We were unable to determine the amino terminal residue of the purified enzyme by peptide sequencing, thus indicating that the amino terminal was blocked. We collected 15 isolated chromatographic peaks from the lysylendopeptidase digest of the purified α-amylase and sequenced them. The resulting sequences were located on a linear sequence deduced from expected homology to the known mammalian α-amylases. Roughly half of the whole sequence was determined from these fragments and the missing sequence was deduced from the nucleotide sequences of DNA clones complementary to the rat liver mRNA for α-amylase. The resulting primary structure was highly homologous, but not identical, to both rat pancreatic and mouse salivary gland α-amylases (Fig. 4). Thus we confirmed that, as in mouse and human, rat had at least two distinct genes for α-amylase isozymes, i.e. the pancreatic and the salivary gland types; in human and mouse, the liver and salivary

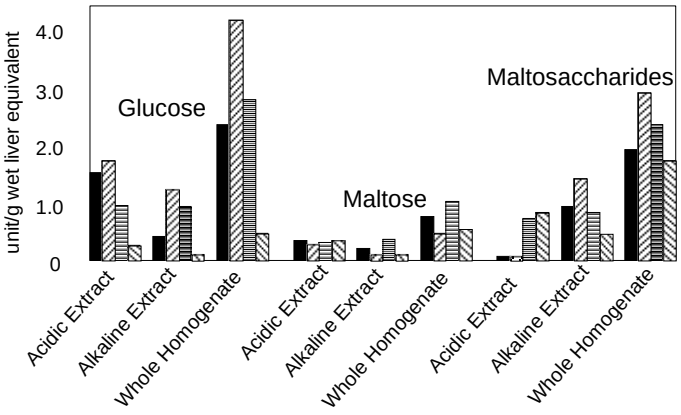

Fig. 3. α-Amylase activities in rat liver. The livers from four rats were separately treated for α-amylase extraction as described in the text. α-Amylase activities in the acidic extracts and the first alkaline extracts are presented in the figure. The total α-amylase activity in the figure was deduced by assuming that half of the activity was extracted in each alkaline extraction. The extraction was repeated 5 times and confirmed the validity of this assumption. Each column represents each rat.

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rat liver        1 MREYCQHHSRMKFFLLLSLIGLCWAQYDPHTFYGRSSIVHLFEWRWVDIA
mouse liver      1 ----------MKFFLLLSLIGFCWAQYDPHTQYGRTAIIHLFEWRWVDIA
rat pancreatic   1 ----------MKFVLLLSLIGFCWAQYDPHTADGRTAIVHLFEWRWADIA
                           ***.*******.********* .**..*.*******.***

    51   KECERYLAPNGFGGVQVSPPNENIVVNSPFRPWWERYQPISYKICSRSGNEDEFRDMVNR
    41   KECERYLAPNGFAGVQVSPPNENIVVHSPSRPWWERYQPISYKICSRSGNEDEFRDMVNR
    41   KECERYLAPKGFGGVQVSPPNENIIINNPSRPWWERYQPISYKICSRSGNENEFKDMVTR
         *********.**.***********....*.**********************.**.***.*

   111   CNNVGVRIYVDAVINHMCGVGAEAGQSSTCGSYFNPNNRDFPGVPYSGFDFNDGKCKTGS
   101   CNNVGVRIYVDAVINHMCGVGAQAGQSSTCGSYFNPNNRDFPGVPYSGFDFNDGKCRTAS
   101   CNNVGVRIYVDAVINHMCGSGNSAGTHSTCGSYFNPNNREFSAVPYSAWYFNDNKCN---
         ******************.*..**..************.*..****...***.**....

   171   GGIENYNDAAQVRDCRLSGLLDLALEKDYVRTKVADYMNHLIDIGVAGFRLDASKHMWPG
   161   GGIENYQDAAQVRDCRLSGLLDLALEKDYVRTKVADYMNHLIDIGVAGFRLDASKHMWPG
   158   GEINNYNDANQVRNCRLSGLLDLALDKDYVRTKVADYMNNLIDIGVAGFRLDAAKHMWPG
         *.*.**.**.***.***********.*************.*************.******

   231   DIKAVLDKLHNLNTKWFSEGSKPFIYQEVIDLGGEAVSSNEYFGNGRVTEFKYGAKLGKV
   221   DIKAILDKQHNLNTKWFSQGSRPFIFQEVIDLGGEAVSSNEYFGNGRVTEFKYGAKLGKV
   218   DIKAVLDKLHNLNTKWFSQGSRPFIFQEVIDLGGEAIKGSEYFGNGRVTEFKYGAKLGTV
         ****.***.*********.**.***.**********....******************.*

   291   LRKWDGEKMAYLKNWGEGWGFMPSDRALVFVDNHDNQRGHGAGGSSILTFWDARLYKMAV
   281   MRKWDGEKMSYLKNWGEGWGLMPSDRALVFVDNHDNQRGHGAGGASILTFWDARLYKMAV
   278   IRKWNGEKMSYLKNWGEGWGFVPTDRALVFVDNHDNQRGHGAGGASILTFWDARMYKMAV
          ***.****.**********..*.*******************.*********.*****

   351   GFMLAHPYGFTRVMSSFHWPRYFENGKDVNDWVGPPNNNGATKEVTINSDSTCGNDWVCE
   341   GFMLAHPYGFTRVMSSYYWPRNFQNGKDVNDWVGPPNNNGKTKEVSINPDSTCGNDWICE
   338   GFMLAHPYGFTRVMSSYRRTRNFQNGKDVNDWIGPPNNNGVTKEVTINPDTTCGNDWVCE
         ****************....*.*.********.*******.****.**.*.******.**

   411   HRWRQIRNMVAFRNVVNGQPFANWWDNGSNQVAFGRGNKGFIVFNNDDWDLSTTLQTGLP
   401   HRWRQIRNMVAFRNVVNGQPFANWWDNDSNQVAFGRGNKGLIVFNNDDWALSETLQTGLP
   398   HRWRQIRNMVAFRNVVNGQPFANWWDNGSNQVAFSRGNRGFIVFNNDDWALSSTLQTGLP
         ***************************.******.***.*.********.**.*******

   471   AGTYCDVISGDKVDGNCTGIKVYVGSDGNAYFSISNSAEDPFIAIHVESKI
   461   AGTYCDVISGDKVDGNCTGIKVYVGNDGKAHFSISNSAEDPFIAIHAESKI
   458   AGTYCDVISGDKVNGNCTGLKVNVGSDGKAHFSISNSAEDPFIAIHADSKL
         *************.*****.**.**.**.*.***************..**.
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Fig. 4. Deduced amino acid sequence of rat hepatic α-amylase and comparison with rat pancreatic and mouse salivary isozymes. Several overlapping cDNAs that cover the whole coding sequence of rat hepatic α-amylase were cloned and sequenced. The resulting sequences were assembled into a unique sequence that stretched from the initiation codon to the polyA tail. Underlining indicates the sequences also determined from peptide sequencing. Asterisks indicate the conserved residues among the three, while dashes are inserted for maximal homology.

gland α-amylases are shown to be the products of the same single gene.

Conclusion

The most noticeable outcome of the present study is the reproduction of the old observations reported by RUTTER & BROSEMER (1961); the rat liver has a high amylolytic capacity and its larger part is associated with membranous structure. Accordingly, we find some sense in the old arguments on the physiological significance of amylolytic glycogenolysis.

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