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Review

Hydrolases acting on glycosidic bonds: chromatographic and electrophoretic separations

Takanori Moriyama*, Hisami Ikeda

Clinical Laboratories, Asahikawa Medical College Hospital, Nishikagura 4-5-3, Asahikawa 078, Japan

Abstract

We describe analyses of unusual human α-amylase, performed in our laboratory and review available methods for amylase study. Electrophoretic and chromatographic methods provide an effective means for the analysis of amylase isoenzymes and unusual amylase. The recent identification of a selective inhibitor and a monoclonal antibody to amylase isoenzyme contributes to rapid routine clinical assays of amylase isoenzymes. However unusual amylases such as variants, macroamylasemia and sialyl salivary-type amylasemia cannot be detected by those conventional methods. The unusual amylases can only be detected by electrophoresis and can be easily characterized by combination study with chromatographic methods. Electrophoretic and chromatographic methods are universal means to validate unusual amylases found in patient sera. Further basal studies are needed to define the roles of salivary amylase in exocrine fluids using those separation techniques.

Keywords: Reviews; Hydrolases; Enzymes

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*Corresponding author.

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1. Introduction

Hydrolases acting on α-1,4 or α-1,6 glycosidic bonds of polysaccharides are known as amylases. Amylases are of referred to as α- or β-amylases or endo- or exoamylases. α-Amylases (α-1,4-glucan 4-glucanohydrolase; E.C. 3.2.1.1) are endoamylases capable of hydrolyzing internal α-1,4-glycoside bonds in large-chain polymers such as starch, glycogen and dextrins, producing maltose, maltotriose and other dextrins [1,2]. α-Amylases are found widely in bacteria, fungi, seeds in leguminous plants and animals including humans. β-Amylases (α-1,4-glucan maltohydrolase; E.C. 3.2.1.2) which attack nonreducing ends of poly-α-1,4-glucosans are found only in plants. In addition, γ-amylases or glycoamylases (α-1,4-glucan glucohydrase; E.C. 3.2.1.3) and isoamylases (glycogen 6-glucanohydrolase; E.C. 3.2.1.68) are found in nature [3].

This paper refers to human α-amylases, featuring chromatographic and electrophoretic methods for separation and their clinical applications. The study of human amylase had already started in the 1800's. Amylase from saliva was first described by Leuchs in 1831 and was later found in serum by Magendie in 1846 and in urine by Cohnheim in 1863 [4]. Amylase quantitation methodology can be classified in the two general groups of amyloclastic assays and saccharogenic assays [5]. Somogyi's amyloclastic method [6] and its later modifications became the most commonly used assay for several decades. In recent years, colorimetric reaction-rate amylase procedures [7–10] were frequently used in clinical laboratories.

In 1964, Norby [11] first demonstrated the electrophoretic separation of pancreatic and salivary isoenzymes and Muus and Vnenchak [12] showed isoenzymic forms of salivary amylase. Excellent reviews concerning chromatographic and electrophoretic analyses of human α-amylase have already been published [2,4,13]. In this paper, we describe analyses of unusual α-amylase experienced in our laboratory, and review available methods for amylase study.

2. Molecular forms of amylase

2.1. Genetic determinants

Two different loci, Amy1 and Amy2, are responsible for the production of salivary and pancreatic amylase, respectively [2]. Both amylase genes, clustered on the short arm of chromosome 1 (p21), were demonstrated by in situ hybridization [14] and somatic cell genetic analysis [15]. Human pancreatic and salivary amylase cDNAs were cloned and sequenced [16–18]. The primary structures of both Amy1 [19,20] and Amy2 [21] are strikingly homologous, not only in exon sequences but also in exon–intron structure. Matsubara and co-workers detected a third type of human α-amylase gene, Amy3, in a lung carcinoid tissue [22,23]. Independently of these works, Gumucio et al. [24] identified two human amylase genes, termed Amy2A and Amy2B, whose coding exons had identical sequences to those of Amy2 and Amy3, respectively. A novel type Amy3 was renamed Amy2B by Yokouchi et al. [25] Amy1 expression in parotid, Amy2A and Amy2B expression in pancreas and expression of Amy2B in human liver were confirmed by Samuelson et al. [26] using ribonuclease protection assay. According to Shiosaki et al. [27], (1) the newly found gene Amy2B can code for an active amylase, (2) this novel enzyme is more similar to pancreatic amylase than salivary amylase and (3) the enzyme shows unique substrate specificity and thermostability. The presence of Amy2 B transcripts in human liver indicates that liver is possibly a source of elevated pancreatic amylase isoenzyme in serum [26].

2.2. Post-translational modifications

About 25–30% of the human α-amylase molecules secreted into the saliva is glycosylated [28–32]. The complete sequence of the oligosaccharide chains of salivary amylase was elucidated by Yamashita et al. [33], who showed that glycosylated amylase contained a single biantennary asparagine-linked oligosaccharide. This glycosylated salivary amylase is called family A while non-glycosylated salivary amylase is called family B [29]. The salivary family B and pancreatic amylases are reported to

be lightly glycosylated while the salivary family A amylase is a heavily glycosylated protein [29,31,32,34]. Each salivary and pancreatic amylase undergoes deaminations [35–37], and thus a family of isoforms is produced in addition to the band of primary gene product. Deamidation refers to the loss of amide group from the amino acids glutamine and asparagine to generate glutamic acid and aspartic acid. This results in a change from an electrically neutral amino acid side chain to one that is negatively charged and causes the protein to exhibit an altered, more anodic migration on electrophoresis or isoelectric focusing [4]. Electrophoretic patterns of isoamylase from normal serum, pancreatic juice and saliva are shown in Fig. 1. A reasonable explanation of post-translational modifications of salivary amylase was recently proposed by Bank et al. [38]. The carbohydrate structures appear to bind to carbohydrate receptors in the liver, accounting for the rapid clearance of the glycosylated salivary amylase from circulation and in turn, its low concentration in serum [33]. Generally family A salivary amylase is not detected in the electrophoretic zymogram of serum samples. Extensive deamination of amylases within cystic fluids were reported [37,39–41]. These cystic amylases are resistant to neuraminidase and can be distinguished clearly from sialyl salivary-type amylases produced ectopically from tumor cells which will be described in Section 5.

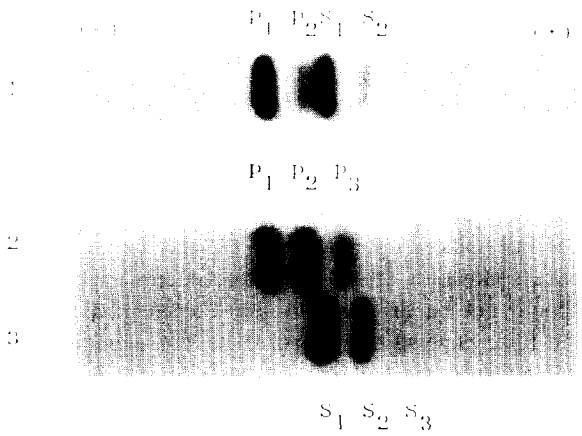

Fig. 1. Electrophoresis on cellulose acetate membrane of isoamylases from normal serum, pancreatic juice and saliva. (1) Normal serum; (2) normal pancreatic juice; (3) normal saliva. Amylase isoenzymes were numbered from the origin toward the anode (e.g., S1, S2,... and P1, P2,...).

2.3. Biochemical and immunochemical properties

The amylase protein is a single polypeptide and most reports estimate its molecular mass to be about 55 000 to 60 000 [31,32,34,40,42]. By SDS–PAGE, the pancreatic and salivary isoenzymes appear to have slightly different molecular mass: the pancreatic isoenzyme migrates faster with a difference in molecular mass of 1000 to 4000. The salivary isoenzyme migrates as two bands with a difference in molecular mass of approximately 1000 to 3000 [4]. The larger molecular mass form is salivary A and the smaller molecular mass form is salivary B. We tried to determine the molecular mass of various amylases by immunoblotting followed by SDS–PAGE [43]. Pancreatic, salivary A and B amylase were prepared from pancreatic juice and saliva, respectively by Superose 12 HPLC according to Bank et al. [38]. Immunoblotting patterns of pancreatic and salivary amylases are shown in Fig. 2. From the data, molecular masses of pancreatic and salivary B amylase were similar and estimated to be approx. 60 000. Salivary A amylase was slightly larger than those.

The p*I* values of pancreatic, salivary A and B amylase were estimated to be 7.0, 6.4 and 5.9, respectively [32,34,44,45]. Amylase is a calcium-requiring metallo-enzyme with at least one calcium tightly bound per protein molecule [46,47]. Chloride is an allosteric activator of amylase [48]. The optimal pH for amylase activity is in the range 7.0 to 8.0 [40,42,49], but can be shifted in the absence of chloride [50].

The amino acid composition of the amylase isoenzymes was determined by several groups [4,28,31,32,40,51]. The pancreatic and the salivary isoenzymes are shown to be quite similar by amino acid composition. Nakamura et al. [16] and Nishide et al. [19] reported that the predicted amino acid sequence homology of the two isoenzymes is about 94% from the study of sequencing of the two cDNAs. The structural similarities between the isoenzymes are in accordance with immunological studies showing extensive cross-reactivities of anti-

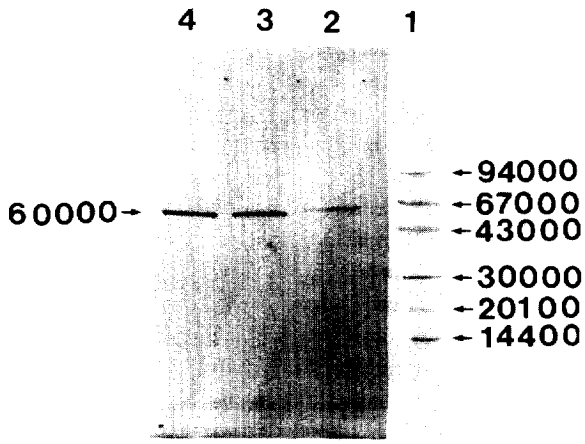

Fig. 2. Immunoblotting patterns of pancreatic, salivary A and B amylase prepared from Superose 12 HPLC. (1); Molecular mass markers: phosphorylase b (Mr=94000), BSA (Mr=67000), ovalbumin (Mr=43000), carbonic anhydrase (Mr=30000), soybean trypsin inhibitor (Mr=20100) and α-lactalbumin (Mr=14400). They were stained with 0.2% Coomassie Brilliant Blue R-250 in 40% methanol–10% acetic acid. (2); Salivary A amylase; (3); Salivary B amylase; (4); Pancreatic amylase. Molecular mass of pancreatic amylase and salivary amylase B were approximately estimated to be 60 000 and of salivary amylase, B was slightly larger than those.

sera against pancreatic and salivary isoenzymes except in a few instances [34,44,52–60]. In later years, antibodies specific to each amylase were developed. Jajaly et al. [61] reported, for the first time, the production of highly selective polyclonal antisera for both salivary and pancreatic isoamylase. Mifflin et al. [62] and Gerber et al. [63,64] reported the production of monoclonal anti-salivary amylase antibody. Immunological determination of serum pancreatic amylase is currently performed in the clinical laboratories using the monoclonal antibody.

3. Electrophoretic methods for amylase

Electrophoresis is one of the most widely used methods for separation of the two principal isoenzmyes and several isoforms of amylase. The principal isoenzymes have been separated with various supports: isoelectric focusing, agar/agarose gel, polyacrylamide gel and cellulose acetate membrane. Each electrophoretic method has its own buffer composition, pH, ionic strength, voltage and/or current requirements and running times. The characteristics of those various methods are summarized in detail in the review by Mifflin et al. [13]. Of the techniques used for separating the amylase isoenzymes, isoelectric focusing offers the highest resolving capability. Acrylamide used for the support may cause some difficulties in handling, staining, scanning and storage. For routine isoamylase electrophoresis, agarose gel film [65,66] or cellulose acetate membrane [67–71] is generally used in the clinical laboratories because of their advantages including easy handling, scanning and permanent storage. Recent technical improvements give satisfactory resolution with cellulose acetate even if not equal to that obtained with acrylamide [43,70,71]. We usually perform amylase electrophoresis with a cellulose acetate membrane, Titan-III lipo (Helena Labs., Beaumont, TX, USA), with Kohn's [72] discontinuous buffer system. Characteristics of cellulose acetate electrophoresis [67–71], including ours [43], are summarized in Table 1. To minimize contamination, we wear latex examination gloves when handling the membrane and placing the wicks in the electrophoresis chamber. Starch and iodine for acrylamide gel [11,38] and dyed-starch conjugates, such as blue starch tablet (Pharmacia, Sweden), for agarose gel [65,66] and cellulose acetate membrane [67–71] are generally used to visualize amylase bands. In blue starch staining procedure on cellulose acetate membrane, careful handling is required to prevent spreading of water-soluble blue dye. We use a slurry of three tablets in 3.5 ml of 8 m*M* $CaCl_2$ in 0.15 *M* NaCl. Immediately after electrophoresis, remaining buffer on both sides of the membrane is blotted off. After pouring the slurry onto the membrane, two sheets of filter paper (Toyo No. 7, Tokyo, Japan) are carefully placed one by one on the surface to remove excess moisture. During these procedures air bubbles should not be inserted between the membrane and the filter papers. After incubation, the membrane is immediately washed out of the slurry with 100% methanol and is soaked in the methanol for 10 min. The membrane is dried with a cold-air dryer and is scanned at 610 nm for quantitation. The membrane can be stored permanently. A method using agarose plates containing blue starch [67,68] is also recommended. We applied this staining method to the

Table 1
Characteristics of cellulose acetate electrophoresis of amylase isoenzymes

Reference	CA membrane	Electrophoresis	Visualization
Takeuchi et al. [67]	Separaphore III (Gelman, USA)	300 V, 180 min 0.02 *M* Phosphate buffer (pH 7.4)	Blue-starch agar (Pharmacia, Sweden)
Benjamin et al. [68]	Sartorius (Sartorius, Germany)	3.5 mA, 45 min Anode: 0.26 *M* Tris–EDTA borate buffer (pH 9.1) Cathode: 0.03 *M* Sodium barbital buffer (pH 8.6)	Blue-starch agar (Pharmacia, Sweden)
Minamimura et al. [69]	Cellogel (Chemtron, Italy)	140 V, 150 min 0.1 *M* Tris–HCl buffer (pH 8.8)	Amyloclastic (Starch/iodine)
Legaz and Kenny [70]	Sartorius (Sartorius, Germany)	3.0 mA, 75 min Anode: 0.15 *M* Tris–borate buffer (pH 9.2) Cathode: 0.03 *M* Sodium barbital buffer (pH 8.6)	Blue-starch slurry (Pharmacia, Sweden)
Massey [71]	Titan-III (Helena, USA)	300 V, 90 min 0.025 *M* Tris–barbital buffer (pH 8.8)	Blue-starch slurry (Pharmacia, Sweden)
Our method [43]	Titan-III (Helena, USA)	300V, 60 min Anode: 0.34 *M* Tris–glycine buffer (pH 9.1) Cathode: 0.05 *M* Sodium barbital–borate buffer (pH 8.9)	Blue-starch slurry (Pharmacia, Sweden)

CA=cellulose acetate; Tris=tris(hydroxymethyl) aminomethane; EDTA=ethylenediaminetetraacetic acid

cellulose acetate membranes for immunoelectrophoresis and thin-layer chromatography of amylase [73]. A convenient staining kit (Nihonsyoji, Tokyo, Japan) is also available. This procedure is based upon a coupled enzyme technique that leads to the production of NADH and finally to accumulation of formazan dye.

The standing Committee on Enzymes of the International Union of Biochemistry has recommended that multiple enzyme forms be numbered starting with the most anodal isoenzyme [74]. While this method may lend itself to some isoenzyme system, it is impractical for human amylase isoenzymes since more anodal isoenzymes appear by deamidation as described previously. It has become standard practice to number human amylase isoenzymes from the origin toward the anode (e.g., S1, S2,.., and P1, P2,..). Normal electrophoretic patterns of pancreatic, salivary and serum amylase isoenzymes are shown in Fig. 1.

4. Chromatographic methods for amylase

Since pancreatic and salivary isoenzymes have essentially similar molecular masses, separation techniques based on gel permeation chromatography are not effective in separating these isoenzymes. Wilding [75] observed a single amylase peak from serum as well as from urine, saliva and pancreatic secretions. An important feature of these chromatographic techniques is the anomalous elution behavior of amylases. During dextran gel permeation chromatography (Sephadex G-75), it was observed that the elution of amylase molecules was considerably retarded, because dextran has a chemical structure similar to the substrate of amylase [76]. We observed the same phenomenon in agarose gel of Superose 12 column matrix [43] and in acrylamide-dextran gel of Sephacryl S-300 [77]. The apparent molecular mass of normal serum amylase was estimated to be approximately 12 500 in Superose 12 gel permeation chromatography [43]. The interaction between the amylase molecule and those gel matrixes can be decreased by adding oligosaccharide in the elution buffer; 1 m*M* β-cyclodextrin or 0.3 m*M* 17-amylose are recommended [78]. So far the two salivary isoenzymes, A and B, have been separated by column chromatography using Bio-gel [28,29,32] and Sephadex [33], concanavalin A affinity chromatography [34] and Superose gel permeation HPLC [38]. For separation of pancreatic and salivary amylase isoenzymes, ion-exchange chromatography utilizing their difference in net electric charge is used. Anion-exchange gels of DEAE-Sephadex

[76,79–81], DEAE-cellulose [79,82] and QAE-Sephadex [28,83,84] are used routinely. Mono Q ion-exchange HPLC has been applied to separate amylase isoenzymes [38,73]. We routinely perform HPLC using a Superose 12 gel permeation column (30 cm×1.0 cm I.D.) and a Mono Q ion-exchange column (5 cm×0.5 cm I.D.) for separation of amylase isoenzymes. The HPLC analysis is carried out on a Pharmacia (Uppsala, Sweden) fast-protein liquid chromatography system. The system was equipped with an LCC-500 gradient programmer, two P-500 dual piston pumps, MV-7 and MV-8 automated injection valves, a solvent mixer, a prefilter, a sample loop, a UV-1 monitor with HR low-dead-volume flow-cell and a Rec-482 recorder. Amylase activity is usually monitored by using an amylase test kit (600 nm, Iatoron Labs., Tokyo, Japan) based on the method of Marshall et al. [85]. The main advantages of the HPLC analyses lie in speed (from 30 min to 90 min), simplicity, small sample requirements and reproducibility of the separation. A separation profile of serum amylase isoenzymes on Mono Q HPLC is shown in Fig. 3. Each isoenzyme can be clearly separated in this way.

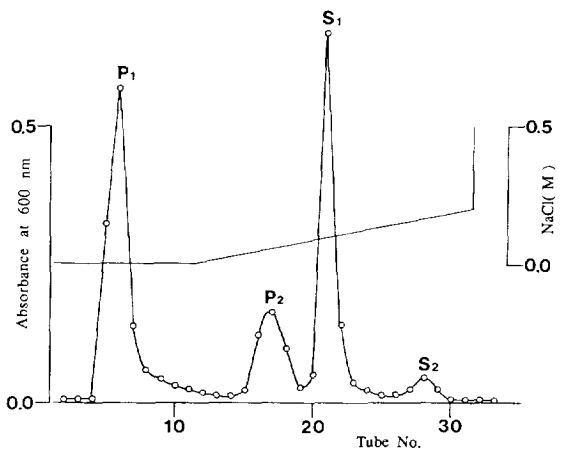

Fig. 3. Separation profile of serum amylase isoenzymes on Mono Q ion-exchange HPLC; 0.5 ml of normal serum amylase in 10 m*M* NaCl, 0.2 m*M* $CaCl_2$, 10 m*M* Tris–HCl buffer (pH 8.5) was injected on a Mono Q column equilibrated with the same buffer. The amylase absorbed in the Mono Q column was eluted with a linear gradient of up to 0.2 *M* NaCl at the rate of 1 ml/min. Amylase activity was monitored at 600 nm.

5. Clinical applications

5.1. Amylase variants

Variants of salivary amylase were first reported by Ward et al. [86] with electrophoretic analysis of saliva samples. The variants were cathodal to the normal components. Pancreatic amylase variants which migrate cathodal to the normal components were first reported by Merritt et al. [87] in samples of pancreatic homogenate. They demonstrated that the alleles of both variants were transmitted in an autosomal codominant mode of inheritance and that the frequencies of the amylase variants were different in ethnic groups. For example, the frequencies of salivary variants are approximately 10% of the white populations and 8% of the black population in the USA [87]. These data are reviewed in detail by Meritt and Karn [2]. Merritt et al. [87] demonstrated pancreatic amylase variants in the serum and urine. Otsuki et al. [88] reported two kinds of pancreatic amylase variants, "slow amylase-1 and dominant amylase-2", in the sera of normal subjects. The slow amylase-1 described by Otsuki et al. [88] is considered to be identical to "Amy2 B and C" reported by Merritt et al. [87]. Dominant amylase-2 was a pancreatic amylase variant which had not been reported previously. Otsuki et al. [89,90] demonstrated that both variant amylases could be detected in the pancreatic juice and the alleles were transmitted in an autosomal codominant mode of inheritance. In Japanese populations, the prevalence of slow amylase-1 was 0.23% [88], which was very low compared with the 10.5% in Caucasian–Americans found by Merritt et al. [87]. So far we experienced only one case with slow amylase-1 in serum (unpublished data) as shown in Fig. 4. Recently, we reported three cases of dominant amylase-2 found in routine electrophoretic analysis of 1691 patient sera [91]. The electrophoretic pattern of a patient serum with dominant amylase-2 is also shown in Fig. 4. K_m value and molecular mass of dominant amylase-2 were the same as that of normal pancreatic isoenzyme [91]. In contrast, Otsuki et al. [89] reported that the prevalence of dominant amylase-2 was 1.68% in normal Japanese subjects. It was suggested that the prevalence of the variants was different

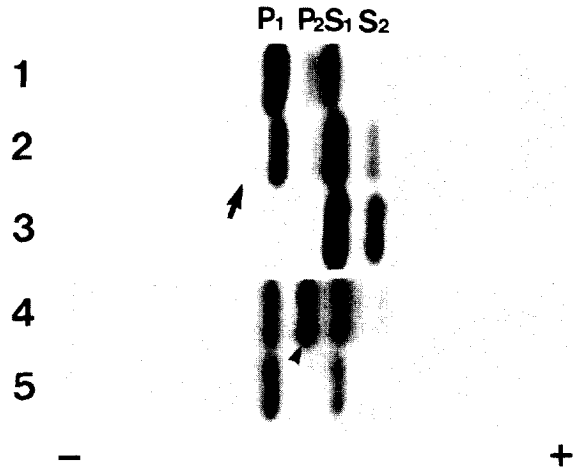

Fig. 4. Electrophoretic patterns of amylase from two patient sera with slow amylase-1 and dominant amylase-2. (2) Patient serum with slow amylase-1 indicated by the arrow, (4) patient serum with dominant amylase-2 indicated by the arrow head, (1,3 and 5) control serum amylase [(1) acute pancreatitis, (3) post-operative salivary hyperamylasemia and (5) normal].

between normal and hospitalized subjects in Japanese populations. Zhaozhou et al. [92] reported an unique new variant pattern of "mixed type" which has a slow amylase-1 and a dominant amylase-2, from the urine samples of 6708 Mongolians of ethnic Chinese descent. The pedigree study revealed that dominant amylase-2 was inherited from father and slow amylase-1 is maternal. In future a novel gene analysis is expected for the study of amylase variants such as serum LDH [93,94] or colinesterase variants [95].

5.2. Hyperamylasemia

Electrophoresis is a first choice to observe the nature of isoenzymes in hyperamylasemia. The isoenzymes can be also distinguished by selective inhibition of the salivary isoenzyme using wheat-germ proteins [96,97] or monoclonal antibody [62,63]. While pancreas and saliva are well-known sources of amylase, amylase activity has also been reported in a variety of other normal tissues and fluids. These include lung, sweat, leukocytes and thrombocytes, colostrum and milk, tears, tonsils, thyroid, liver, bile, endometrium, semen, falopian tubes, amniotic fluid, cervical mucosa, intestinal mucosa, prostate gland and seminal vesicles [4]. The existence of amylase in liver was controversial for many years [2]. However, at present, expression of Amy2B in liver is confirmed by Samuelson et al. [26]. While tissues and fluids except liver and bile are now thought to contain salivary type, further electrophoretic characterization is necessary in future. Amylase is secreted into numerous fluids that moisten body surfaces exposed to the external environment, raising the possibility that amylase may have some role in host defense mechanisms [4].

Pancreatic isoenzyme has been demonstrated only in pancreas and its secretions. A patient with pancreatitis usually has elevated pancreatic type amylase which returns to normal on clinical improvement [13,98]. In acute pancreatitis, a large increase of pancreatic amylase sub-band was disclosed and its average half-time activity was about 10 days [70,99]. The content of the pancreatic amylase sub-band is used as a useful aid for differential diagnosis of acute pancreatitis in patients exhibiting equivocal hyperamylasemia [100–102]. Dominant salivary isoenzymes were detected routinely in mumps-induced hyperamylasemia and hyperamylaseuria [2,67]. Salivary-type hyperamylasemia was also generated in a variety of cancers including pancreas [103], lung [104,105], ovarian [106,107], stomach [108] and uterus [109] cancers. There was an interesting report that a patient with primary pancreas cancer showed hyperamylasemia due to salivary-type isoamylase [102]. Shimamura et al. [110] demonstrated that normal pancreatic extracts contain some salivary isoamylase. Recent biochemical studies have shown that tumour-associated amylases are mostly of the salivary type. In contrast, some authors reported pancreatic-type isoamylase was produced in one case of uterine cancer [111] and in two cases of breast cancer [112,113]. Most of the amylases are produced by epithelial tissues. Recently, two cases of non-epithelial osteosarcoma [114] and multiple myeloma [115] tumours with amylase production were reported. The amylases were also of the salivary type.

Post-operative transient hyperamylasemia [116–118] is often encountered in routine serum amylase analyses. Most of the amylases are of the salivary

type but their origin is not well known. Hyperamylasemia associated with a cerebral trauma [119], an injury [120] and a ruptured ectopic pregnancy [121] were also reported. Persistent hyperamylasemia is known to occur in patients with end-stage renal diseases [122–127]. This type of hyperamylasemia is caused by impaired excretion of the pancreatic isoamylase by the damaged kidney [125,126]. Another type of persistent hyperamylasemia occurs in patients with macroamylasemia.

5.3. Macroamylasemia

In 1964, abnormally large serum amylase, found in the serum of a patient with persistent hyperamylasemia, was identified by Wilding et al. [128]. Berk et al. [129] reported three cases of large-sized amylase and suggested the designation "macroamylase" as a descriptive term for this condition. Levitt and Cooperband [130] demonstrated, by the immunoglobulin precipitation method, that the macroamylase was an immune complex resulting from the binding of normal amylase and IgA. Levitt et al. [131] reported that the high serum amylase levels found in the patients with macroamylasemia were associated with an extremely low ratio of *Cam/Ccr*, in contrast to the situation in acute pancreatitis. This *Cam/Ccr* ratio is a useful indirect feature of macroamylasemia and still used in its detection. However, Berk et al. [132] reported that the ratio was markedly decreased in the patient sera with salivary-type hyperamylasemia. The low ratio of *Cam/Ccr* in hyperamylasemia dose not necessarily mean macroamylasemia. Levitt et al. [131] demonstrated some important characteristics of the amylase-IgA complex: (1) the remaining amylases in serum, intestinal fluid, urine and saliva were of normal size; (2) the complex dissociated at acidic pH; (3) the IgA isolated by acidic dissociation bound with normal serum amylase; and (4) macroamylase resulted from the binding of normal amylase by an abnormal IgA in a unique autoimmune reaction. This report was of great significance in the study of macroamylase and other macroenzymes [133]. Fridhandler et al. [134,135] showed, from the study of isoamylases dissociated from the complexes by acidification, that the binding substances displayed more or less greater affinity for salivary isoamylase. We also recognized similar tendencies for the immunoglobulins in the complexes [77]. We reported a unique case of pancreatic amylase–IgA complex [136] and of salivary amylase–IgG complex [73]. The electrophoretic patterns of patient sera with macroamylasemia are peculiar, generally broad as first reported by Harada et al. [137]. The typical electrophoretic patterns of patient sera with macroamylasemia, experienced in our laboratory, are shown in Fig. 5 [138]. The zymograms of our cases showed a characteristic broad pattern which could not be separated into pancreatic and salivary isoamylase bands. The broad bands started from the various positions of P1, P2, S1 and S2 subband and were of various widths. Tozawa [139] classified 127 specimens of macroamylase into eight groups and four further general groups depending on the location of the typical bands obtained with cellulose acetate membrane electrophoresis. He suggested that the broad bands of IgG complex were different from those of IgA complex. Kanno and Sudo [140] were the first to report that the binding site of amylase linked IgG was located in the Fab portion of the IgG molecule and that the macroamylases were specific antigen–antibody complexes. We also demonstrated that the amylase molecule was associated with the Fab portion of the IgA [141] and IgG [73] molecule in the amylase–Ig complexes. Harada et al. [137]

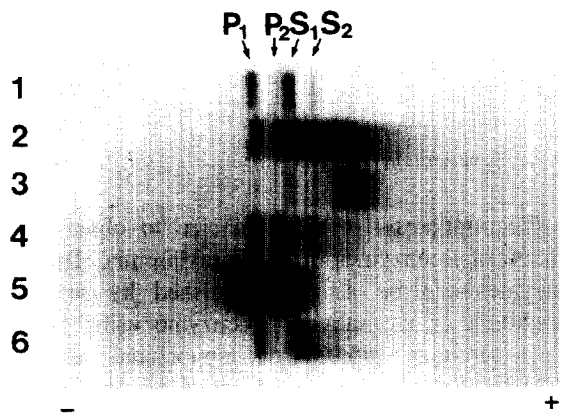

Fig. 5. Electrophoretic patterns of amylase from patient sera with macroamylasemia. (1) Normal serum, (2) IgA-λ type, (3) IgA-κ type, (4) IgA-κ, λ type, (5) IgG-κ type, (6) IgA-λ type. A characteristic broad band started from the various positions of P1, P2, S1 and S2 subband and were of various width.

and Kanno and Sudo [140] applied a immunoelectrophoresis technique following amylase staining to the direct identification of amylase–Ig complexes in the patient sera. For the routine detection of macroamylase, a peculiar broad band found by electrophoresis is tested and the presence or absence of macromolecular amylase peak is confirmed by gel permeation HPLC. Then identification of the binding immunoglobulin is performed by immunoelectrophoresis [137,140], electrophoresis [142,143], immunofixation counter current electrophoresis [144,145] or immunoprecipitation reaction [146]. Frequently we use both counter current electrophoresis and immunoprecipitation. The former method can simultaneously demonstrate the electrophoretic pattern and binding immunoglobulins, as shown in Fig. 6. The latter is the most sensitive and a convenient method.

So far, we have experienced 39 cases of macroamylasemia and identified the binding immunoglobulins in all cases. Of these cases, 23 (59.9%) were in males and 16 (40.1%) were in females. The age ranged from 9 to 81 years but most patients (67.7%) were more than 50 years old. Total amylase activity, at the discovery, ranged from 142 to 2222 U/l and 37 cases out of 39 (94.9%) were above the upper value of normal range (175 U/l). The mean and standard deviation were 519 U/l and 392.9 U/l, respectively. Almost of all cases showed a persistent hyperamylasemia due to macroamylasemia but we observed one case with transient macroamylasemia in a 10-year-old girl who had been treated with valproic acid for epilepsy since the age of 5 years [147]. The classes and types of immunoglobulins of the macroamylases are shown in Table 2. IgA-type macroamylases were dominant and consisted of 82.1%. There was no difference between κ or λ type immunoglobulins. Our data were similar to the results of Tozawa's [146] mass screening. The molecular mass estimated in 32 cases ranged from 220 000 to 470 000. The mean and the standard deviation were 302 000 and 62 000, respectively. Consequently, it was suggested that the complexes usually consisted of one molecule of immunoglobulin and two molecules of amylase; the lowest molecular complex was of the ratio of 1:1. Ueda et al. [148] reported that one case exhibited a sedimentation coefficient greater than 19S serum protein, from an ultracentrifugal studies of 22 patients. They did not identify the binding immunoglobulins but it was suggested that the macroamylase consisted of IgM or plural IgA or IgG molecules. Clinical diagnoses of the patients included a wide variety of diseases and symptoms. In our 39 patients, diseases of the digestive system (30.8%) were most frequent and malignancies (20.5%) were the second. Three patients had an autoimmune disease (7.7%): ulcerative colitis, systemic lupus erythematosus and Sjögren's syndrome. Five patients without symptoms (12.8%) except hyperamylasemia were observed. Our cases with macroamylasemia did not show

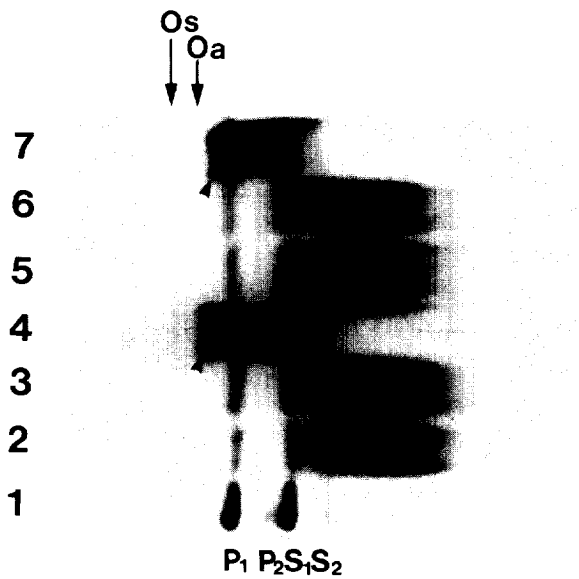

Fig. 6. Counter current electrophoretic pattern of a patient serum with IgA-λ type macroamylasemia. Os, Origin of normal patient serum, (PS); Oa, origin of antisera; (1) normal serum, (2) PS, (3) PS, anti-IgG serum, (4) PS, anti-IgA serum, (5) PS, anti-IgM serum, (6) PS, anti-L-κ serum, (7) PS, anti-L-λ serum. Amylase activities were detected on the precipitation lines indicated by the arrow heads (lane 4 and 7).

Table 2
The classes and types of immunoglobulins of the macroamylases

	κ	λ	κ–λ	Total
IgA	11 (28.8%)	15 (38.5%)	6 (15.4%)	32 (82.1%)
IgG	5 (12.8%)	1 (2.6%)	0 (0.0%)	6 (15.4%)
IgG-A	1 (2.6%)	0 (0.0%)	0 (0.0%)	1 (2.5%)
Total	17 (43.6%)	16 (41.0%)	6 (15.4%)	39

association with any particular disease or symptom complex, as in other reports [146,149]. Tozawa [146] reported that macroamylasemia was found in 0.18% of hospital patients and 0.04% of blood donors and the incidence in males was significantly ($P<0.01$) greater than in females. Coincidence of macroamylasemia and other serum macroenzymes was reported in a patient serum with rheumatoid arthritis by Maekawa et al. [150], and in two patient sera by Tozawa [146]. In the mass screening study of macroenzymes, Tozawa [146] reported two rare coincidence cases with amylase–Ig complex and LDH–Ig or CK–Ig complex. Recently, Zaman et al. [151] reported a case of the simultaneous presence of macroamylasemia and macrolipasemia in a patient with celiac disease. At present, a macroamylase is thought to be a antigen–antibody complex as other macroenzymes [139,152–155] produced by autoimmune reaction. The affinity constants of antibodies involved in macroamylases calculated by Urdal et al. [156] were 10^{-9} to 10^{-10} mol/l which is in the range of other high-affinity antigen–antibody complexes. Hortin et al. [157] reported a novel ELISA method detecting macroamylase and free autoantibody to amylase. With the ELISA, free antibody to amylase was positive in 11 (33.3%) of 33 samples known to contain macroamylase.

The major clinical importance of macroamylase lies in its potential to create confusion during the investigation of possible pancreatitis. At present, convenient commercial kits of wheat-germ [158] and monoclonal inhibition [64] methods are available to analyze amylase isoenzyme distribution. However, it should be noted that both methods failed to detect macroamylasemia. Namely, wheat-germ and monoclonal antibody can not adequately react to salivary amylase in the macroamylase because immunoglobulin is binding. Then the inhibition methods with wheat-germ [159,160] and monoclonal antibody [161,162] were obtained resulting in a high proportion of pancreatic amylase by incomplete inhibition of salivary amylase. It is also important to analyze the total amylase activity of macroamylasemic sera. It was reported that macroamylasemic sera showed significant differences in activity against various-sized substrates [163,164] and a kind of autoanalyzer was developed [165]. Electrophoretic and chromatographic methods are indispensable techniques to characterize macroamylasemic sera.

Macroamylase that were not an immune complex were reported [166–170]. Those macroamylases were complexed with substances such as hydroxyethyl starch (HES). Various species of HES have been developed for use in the management of hypovolemia and intravenous infusion of HES results in iatrogenic macroamylasemia. Tozawa et al. [171] were the first to demonstrate electrophoretic patterns of patient sera with HES-induced macroamylasemia. Namely, the electrophoresis of the sera revealed anomalous patterns with cathodally migrating broad amylase bands. The electrophoretic pattern and the Superose 12 HPLC elution profile of HES-induced macroamylasemia are shown in Fig. 7. Macroamylase complexed with the HES has rarely been

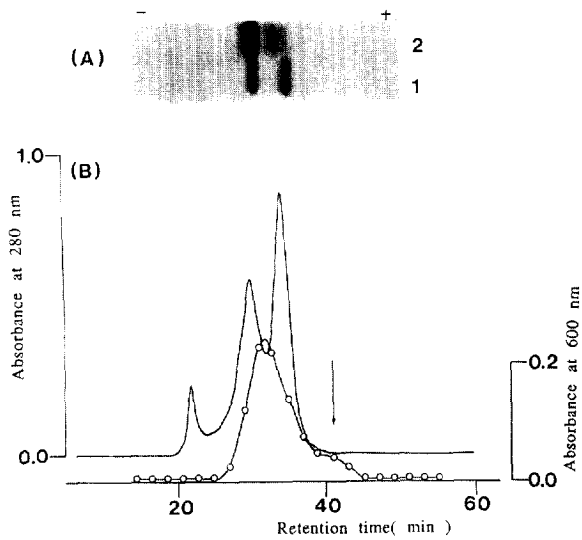

Fig. 7. Electrophoretic pattern and Superose 12 HPLC elution profile of amylase from a patient serum with macroamylase induced by HES. (A) Electrophoretic pattern. (1) Normal serum amylase, (2) patient serum 2 h after termination of the infusion of the HES (Hespander, Kyorin Pharmaceutical, Tokyo, Japan). The patient serum revealed an anomalous pattern with cathodally migrating broad bands. (B) Superose 12 HPLC elution profile; 0.2 ml of the same patient serum was injected and eluted at 0.4 ml/min with 0.15 *M* NaCl, 0.05 *M* phosphate buffer (pH 7.2). The protein absorbance (—) and the amylase activity (○) were monitored at 280 nm and 600 nm, respectively. The elution position of normal serum amylase is indicated by the arrow.

found in clinical laboratories. Accordingly, the descriptive term "macroamylase" is now mostly applied to amylase–immunoglobulin complexes.

5.4. Sialyl salivary-type amylasemia

In the many reports describing tumour-associated amylase, Sudo and Kanno were the first to find sialic acid-containing amylase in the patient sera of pancreatic cancer and lung cancer [172,173]. The amylase isoenzyme in the patient sera showed an abnormal anodic migration by an electrophoretic technique. The abnormal amylase was named sialic acid-containing amylase because of its sensitivity to neuraminidase [172]. Nakayama et al. [105] also reported the same type of amylase in lung cancer. We were the first to report the sialic acid-containing amylases associated with IgA-type myeloma [43] and with IgD-type myeloma [174]. Their phenotypes were salivary-type. This neuraminidase sensitive fast-migrating amylase was named a sialyl salivary-type amylase [174]. The electrophoretic and Superose 12 HPLC elution pattern of a serum sample of sialyl salivary-type amylasemia with IgD-type myeloma, are shown in Fig. 8. The sialyl salivary-type amylase could be separated clearly from residual normal isoamylases of patient sera [43,174] by electrophoresis and HPLC. In eight reported cases of amylase-producing myeloma [115,175–181], fast-migrating isoamylase was clearly recognized in one case [178]. The case was an IgD-type myeloma possibly with salivary-type because its migration pattern was similar to that of our cases. Sandiford and Chiknas [182] found fast-migrating amylase in the serum of a patient with advanced ovarian cancer but its properties were not studied in detail. It is possible that their amylase is sialyl salivary-type amylase because its migration pattern was also similar to that of our cases. We found the same cases of sialyl salivary-type amylasemia with ovarian cancer (unpublished data). It should be noted that acidic amylase from ovarian cystic fluids [39,40] and lymphoepitherial cyst [41] are clearly different from the sialyl salivary-type amylase. These cystic amylases are unaffected by treatment with neuraminidase. Therefore, these amylases are thought to result from aging transformation of cystic amylase, as

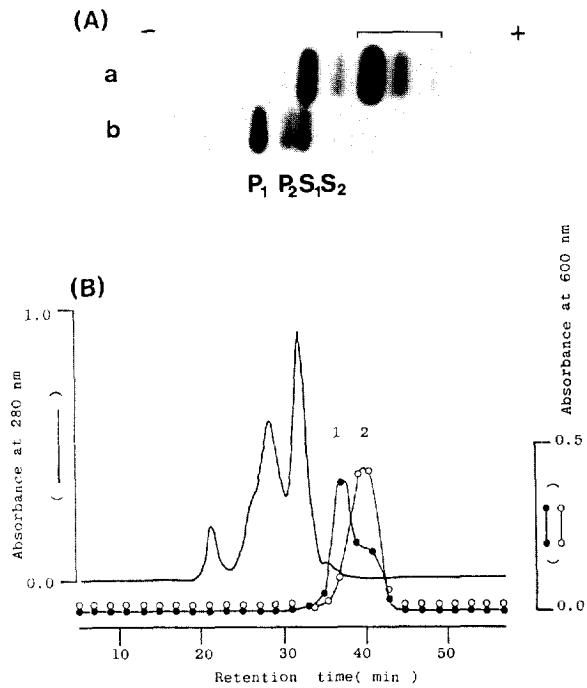

Fig. 8. Electrophoretic pattern and Superose 12 HPLC elution profile of amylase from a patient serum with sialyl salivary amylasemia, suffering from IgD-myeloma. (A) Electrophoretic pattern. (a) Patient serum, (b) normal serum. The fast-migrating isoamylases, indicated by parenthesis, were detected in the patient serum. (B) Superose 12 HPLC elution profile; 0.2 ml of the same patient serum was injected and eluted at 0.4 ml/min with 0.15 *M* NaCl, 0.05 *M* phosphate buffer (pH 7.2). The protein absorbance (—) and the amylase activity (●○) were monitored at 280 nm and 600 nm, respectively. (—) Patient serum protein, (●) patient serum amylase, (○) normal serum amylase. Peak 1 and 2 of patient serum contained the fast-migrating isoamylases and residual normal isoamylases, respectively.

reported by Warshaw and Lee [36] and Weaver et al. [37]. Otsu et al. [183,184] histochemically showed amylase activity in the thyroid gland. Electrophoresis of the amylase in the thyroid gland showed essentially salivary amylase band and an unknown anodic band. The anodic amylase band may be sialyl salivary-type amylase.

According to Yamashita et al. [33] glycosylated salivary isoamylase from the parotid gland contains single asparagine-linked sugar chains in one molecule. Yamashita et al. [185] reported that the sugar

chains of tumour-derived amylases completely lacked the fucosyl residue. Takeuchi et al. [186] reported that tumour amylases differ from normal salivary amylase in amino acid composition. The sugar chains of sialyl salivary-type amylase in our cases [43,174] had no affinity to Con A, as normal serum amylase, while tumour amylase reported by Sudo and Kanno [173] had a strong affinity to Con A. These results suggest that there is micro-heterogeneity in the sugar chains of the tumour amylases.

6. Biological fluids

6.1. Urine

Analysis of amylase isoenzymes in the urine is routinely applied to auxiliary diagnosis of hyperamylasemia. Experimental studies on animal and man have demonstrated that amylase is first filtered by the glomeruli after which, regardless of the size of the load, about 45–70% is reabsorbed by the tubules [187–190]. Recently, studies of urinary amylase isoenzymes are also carried out in diabetes mellitus [191,192]. Both salivary and pancreatic amylases have identical molecular size but different net charges in plasma. Salivary amylase is more anionic (p*I* 5.9–6.4) than pancreatic amylase (p*I* 7.0) [34,44,45]. Pancreatic amylase is more easy excreted than salivary amylase in the urine of normal subjects [193]. It has been shown that their excretion rates in urine are altered in several nephropathies [194], probably due to loss of the negative charges normally present in the glomerular basement membrane. Recio et al. [191] reported that altered S/P ratios appeared more frequently than microalbuminuria in population studies of diabetes mellitus.

6.2. Others

Amniotic fluid contains amylase, the level of which gradually increases up to 36 weeks gestational age and then abruptly stops. There is no correlation between amylase levels in the maternal serum and the amniotic fluid [195]. The S/P ratio in the amniotic fluid was highly correlated with fetal age [196].

Singh [197] recently reported a unique study concerning uterine fluid amylase. He demonstrated that in the uterine fluid of infertile women the amylase activity was significantly lower than that in parous women during the different phases of menstrual cycle. He speculated that the low enzyme activity in infertile women could impair the sperm capacitation and thus adversely affects the fertility of these infertile women. He did not mention the phenotype of the amylase but salivary-type is presumed. Salivary amylase is secreted into numerous fluids that moisten body surfaces exposed to the external environment. This study is a very interesting with respect to the role of salivary amylase in exocrine fluids. Many studies on amylase in various organs or biological fluids measured only amylase activity and did not assay for specific isoenzymes.

At present we are interested in myeloma associated ectopic amylase production. Those amylase were all salivary-type. In 1983, Zakrzewska and Prokopowicz [198] reported the detection of amylase activity in leukocytes of patients with leukaemia as well as in normal subjects. They described the enhancement of amylase activity as being correlated with the rate of leukocyte maturation. The phenotype of amylase in leukocytes is salivary-type, as reported by Vacíková [199]. Therefore, the production of amylase by myelomas is regarded as elevated amylase production by lymphocyte transformation rather than as ectopic production. The role of amylase as a host-defense mechanism has been postulated from its production in human leukocytes and thrombocytes, where it may function during phagocytosis of microorganisms [199].

7. List of abbreviations

Amy1	Gene of amylase 1 (salivary amylase)
Amy2	Gene of amylase 2 (pancreatic amylase)
BSA	Bovine serum albumin
cDNA	Complementary DNA
Cam/Ccr	Ratio of amylase clearance to creatinine clearance
CK	Creatine kinase
Con A	Concanavalin A
DEAE	Diethylaminoethyl
ELISA	Enzyme linked immunosorbent assay

Fab	Fab fragment of immunoglobulin molecule (Fragment of antigen binding)
HES	Hydroxyethyl starch
HPLC	High-performance liquid chromatography
Ig	Immunoglobulin
K_m	Michaelis constant
LDH	Lactate dehydrogenase
L-κ	Light chain κ
L-λ	Light chain λ
NAD	Nicotinamide adenine dinuclecotide hydrogen
P	Probability
pI	Isoelectric point
PAGE	Polyacrylamide gel electrophoresis
QAE	Quaternary aminoethyl
SDS	Sodium dodecyl sulphate
S/P	Ratio of salivary amylase to pancreatic amylase
Tris	Tris(hydroxymethyl)aminomethane

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