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Separation and characterization of sialic acid-containing salivary-type amylase from patients' sera with immunoglobulin A-type myeloma

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ABSTRACT

Isoamylases, with an abnormal anodic migration, were detected by an electrophoretic technique in the sera from two patients with immunoglobulin A-type myeloma. The abnormal isoamylase bands migrate towards the anode faster than the salivary isoamylase (S_2) band and were stained more strongly than the S_2 sub-band. The abnormal isoamylase could be separated from patients' sera by using size-exclusion high-performance liquid chromatography. The serum abnormal isoamylases were showed to be sialic acid residues containing amylase, after the study of treatment with neuraminidase (EC 3.2.1.18), and to be salivary-type amylase, after the study of reaction with human salivary monoclonal antibody. The abnormal bands were not detected in the saliva from one patient. The two patients had no detectable malignancies except myeloma. These findings strongly suggest that the sialic acid-containing salivary-type amylases were produced ectopically from myeloma cells. In this regard the ectopic amylase production by myeloma cells is discussed.

INTRODUCTION

Ectopic production of amylase has been reported in a number of tumours, including lung [1,2], ovary [3,4], stomach [5], pancreas [6] and uterus [7]. In these cases most of the amylases are produced by epithelial tissues. Recently, two cases of non-epithelial tumours with amylase production constituted osteosarcoma [8] and immunoglobulin A (Ig A)-type multiple myeloma [9] have been reported. It has been demonstrated that the amylases, which are ectopically produced, are of salivary type [1–6].

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In 1975, Sudo and Kanno [10] reported a very rare case with ectopic amylase production. This serum amylase had an unusually fast electrophoretic mobility, which was due to the presence of sialic acid residues in its composition.

This paper described the detection of isoamylases, with abnormal electrophoretic behaviour, in the sera from patients with IgA-type myeloma. These abnormal serum amylases were separated by size-exclusion high-performance liquid chromatography (HPLC) and were then characterized, in comparison with salivary amylase, by an electrophoretic isoenzymic technique.

EXPERIMENTAL

Serum samples

Serum samples were obtained from the following two cases. Case 1, a 85-year-old woman, was an outpatient in Asahikawa Medical College Hospital. A hyperamylasemia (1836 U/l, normal range 38–175 U/l), a monoclonal proteinemia, and a normocytic and normochromic anemia (haemoglobin 6.8 g/dl) were identified in laboratory tests on the first medical examination. Total protein was 8.3 g/dl. Serum immunoglobulin concentrations were: IgG, 550 mg/dl; IgA, 4050 mg/dl; and IgM, 43 mg/dl. Bence–Jones protein was not found in her urine. Myeloma was presumed from these laboratory tests. The amylase isoenzyme analysis by electrophoresis showed an abnormal pattern.

Case 2, a 70-year-old man, was an inpatient in the Hyogo Medical College Hospital and was treated for renal failure in typical multiple myeloma. Laboratory data at the admission were as follows: total protein, 8.5 g/dl; IgG, 479 mg/dl; IgA, 418 mg/dl; IgM, 9 mg/dl; haemoglobin, 5.5 g/dl; and serum amylase activity, 1554 U/l. A monoclonal protein (M-protein) and Bence–Jones protein were found in his serum and urine, respectively. The amylase isoenzyme analysis by electrophoresis showed an abnormal pattern.

Whole saliva and urine

Whole saliva and urine were obtained from case 1. The whole saliva was diluted with an equal volume of isotonic saline solution to reduce the viscosity, and was then centrifuged at 4000 *g* for 30 min to remove debris.

Measurement of amylase activity

Total amylase activity was measured as described previously [11].

Serum protein electrophoresis

A 1% agarose gel plate (Paragon SPE kit, Beckman) was used, and electrophoresis was carried out on a Paragon electrophoresis cell (Beckman), according to the kit manual. The gel was stained with 0.5% Amido Black 10B in 5% acetic acid.

Isoamylase electrophoresis

Cellulose acetate membrane (Titan III lipo, Helena Labs., Beaumont, TX, USA) was used with a discontinuous buffer system, as described by Kohn [12]. Amylase activity was detected by blue starch, according to Leclerc and Forest's technique [13].

Size-exclusion HPLC

HPLC analysis was carried out on a Pharmacia (Uppsala, Sweden) automated fast-protein liquid chromatography (FPLC) system with a Superose 12 HR column (30 cm × 1.0 cm I.D.) as described previously [14]. Protein was monitored at 280 nm, and amylase activity was monitored with use of an amylase test kit (Iatoron Labs., Tokyo, Japan) based on the method of Marshall *et al.* [15]. For the determination of the molecular mass of amylase, the following standard proteins (Pharmacia) were used: bovine serum albumin (BSA) (M_r = 67 000), ovalbumin(M_r = 43 000), chymotrypsinogen A (M_r = 25 000) and ribonuclease A (M_r = 13 700).

Concanavalin A high-performance liquid affinity chromatography (Con A HPLAC)

Con A HPLAC analyses were performed on the FPLC system with Con A column (15 cm × 0.46 cm I.D.) purchased from Hohnen (Tokyo, Japan) and carried out according to the method of Takeuchi [16].

Treatment with neuraminidase

Neuraminidase treatment of the proteins was carried out according to the method of Sudo and Kanno [10]. The neuraminidase from *Arthrobactor ureafaciens* was purchased from Nakarai (Kyoto, Japan). Samples were incubated with neuraminidase at 37°C for 1 h.

Immunological study of separated abnormal isoamylases

Inhibitory monoclonal antibody against human salivary amylase was obtained from an Isoamylase PNP kit (Boehringer Mannheim, Tultzing, Germany) based on the method of Gerber *et al.* [17]. The monoclonal antibody binds specifically to salivary amylase and inhibits *ca.* 90% of the total activity. The separated abnormal isoamylases were mixed with this antibody, and the total amylase activities were measured kinetically using the PNP kit with a Gilford Stasar III spectrophotometer (Gilford Instruments, Oberlin, OH, USA). The electrophoresis of the mixture was also performed.

Preparation of salivary amylase

Salivary amylase was purified from human whole saliva with a Mono Q column (5 cm × 0.5 cm I.D., Pharmacia) according to the method reported previously [11].

Immunoblotting

Determination of the molecular mass of abnormal isoamylases was performed by electrophoresis in a 4–20% sodium dodecyl sulphate (SDS) polyacrylamide gradient slab gel (Tefco, Nagoya, Japan) according to the method of Laemmli [18].

Immunoblotting was carried out according to the method of Towbin *et al.* [19]. Proteins were transferred electrophoretically to a nitrocellulose sheet (GV, Millipore, Bedford, MA, USA) in a Funakoshi Model 88 blotting cell (Tokyo, Japan) for 1 h at 180 mA. The lane of standard markers was then separated. The rest of the sheet was soaked in 3% bovine serum albumin (BSA) in 150 m*M* sodium chloride–60 m*M* phosphate buffer (pH 7.2) (phosphate-buffered saline, PBS), then the sheet was incubated in rabbit anti-amylase antiserum (which was diluted 200-fold in 3% BSA–PBS, MBL Labs., Nagoya, Japan) for 2 h at room temperature. Bound antibodies were visualized by avidin–biotin peroxidase complex reagents (Vector Labs., Burlingame, CA, USA) as described by Hsu *et al.* [20].

The following standard markers (Pharmacia) were used: phosphorylase b (M_r = 94 000), BSA (M_r = 67 000), ovalbumin (M_r = 43 000), carbonic anhydrase (M_r = 30 000), soybean trypsin inhibitor (M_r = 20 100) and α-lactalbumin (M_r 14 400). They were stained with 0.2% Coomassie Brilliant Blue R-250 in 40% methanol–10% acetic acid.

RESULTS

The electrophoretic patterns of the patients' serum proteins in agarose gel are shown in Fig. 1A. M-proteins in the β-1 region (case 1) and in the β-2 region (case 2) were detected. These M-proteins were identified as IgA-λ type in case 1 and IgA-κ type in case 2 by an immunofixation technique (data not shown).

Electrophoresis on cellulose acetate membranes of isoamylases in patients' sera is shown in Fig. 1B. Abnormal bands, which unusually migrated faster than the S_2 sub-band of salivary amylase, were demonstrated in both cases by using an amylase acitivity detection method. Both major bands of these abnormal isoamylases were more strongly stained than each S_2 sub-band. In case 1, the abnormal bands were found by densitometry to be 64.1% of the total amylase activity, and the normal bands were 35.9% (P, 2.5%; S_1, 19.6%; S_2, 13.8%). In case 2, the abnormal bands were 41.1% of the total amylase and the normal bands were 58.9% (P_1, 12.6%; P_2, 10.1%; S_1, 20.1%; S_2, 16.1%). Control serum indicated the normal bands (P_1, 33.2%; P_2 12.0%; S_1 39.1%; S_2, 15.8%). Isoamylase electrophoresis of urine and whole saliva from case 1 was also performed, and is shown in Fig. 1C. The electropherogram of the saliva showed a normal pattern. However, the same abnormal bands were detected in the urine from case 1.

The patients' sera were subjected to HPLC using a Superose 12 column. The elution patterns are shown in Fig. 2. The elution peak of normal serum amylase

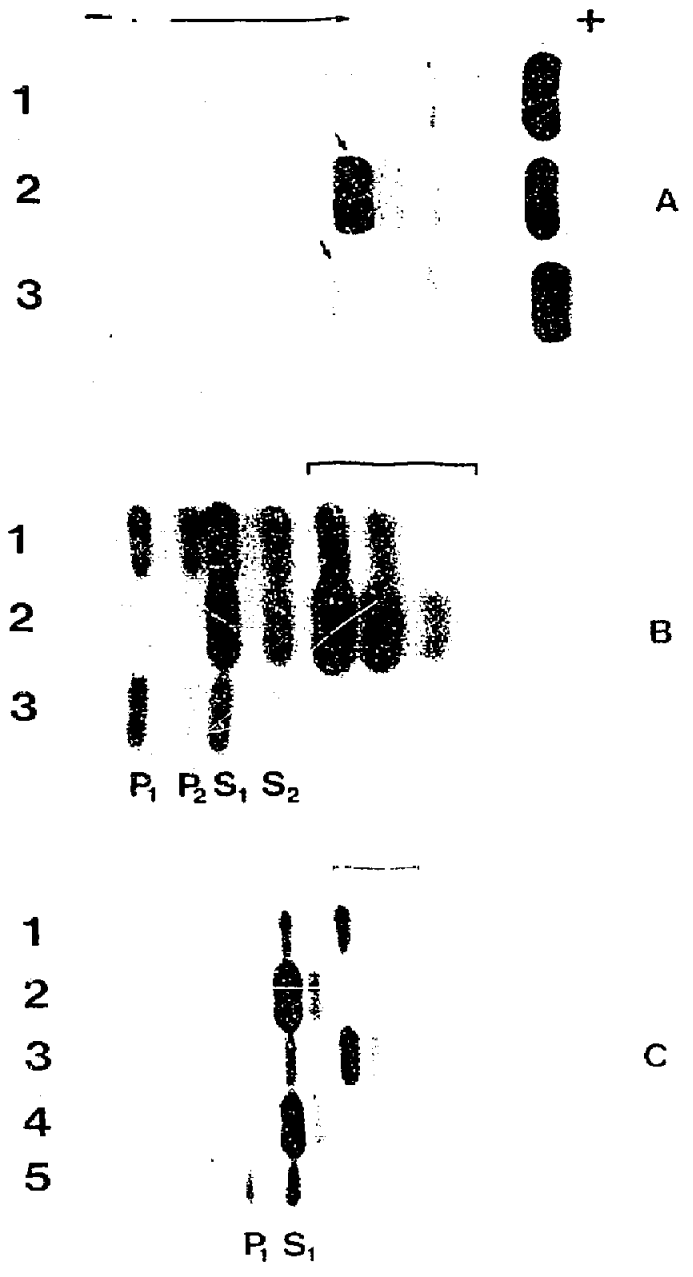

Fig. 1. (A) Agarose gel electrophoresis of patients' serum proteins. The gel was stained with 0.2% Amido Black 10B. 1, Control serum; 2, case 1 serum; 3, case 2 serum. Monoclonal proteins are indicated by arrows. (B) Electrophoresis on cellulose acetate membrane of the serum isoamylase from patients' sera. The amylase activity was detected by the blue starch staining technique. 1, Case 2 serum; 2, case 1 serum; 3, control serum; P_1 and P_2, pancreatic isoamylase; S_1 and S_2, salivary isoamylase. Fast-migrating isoamylases are observed in lanes 1 and 2, indicated by a bracket. (C) Electrophoresis of isoamylase from the urine and whole saliva from case 1. 1, Patient's urine; 2, patient's saliva; 3, patient's serum; 4, control saliva; 5, control serum; P_1 and S_1, pancreatic and salivary isoamylase, respectively. The fast-migrating isoamylases, indicated by a bracket, were detected in the urine but not in whole saliva.

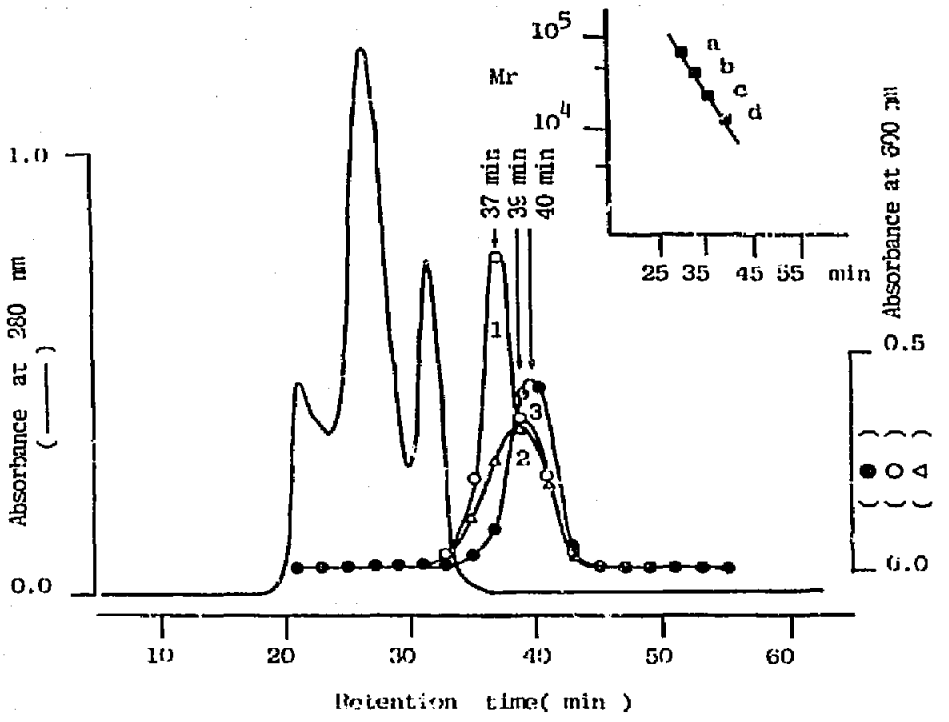

Fig. 2. Elution patterns of the abnormal isoamylases from the patients' sera by HPLC on a Superose 12 column; 0.2 ml of patients' sera was injected and eluted at 0.4 ml/min with 150 m*M* sodium chloride–50 m*M* phosphate buffer (pH 7.2). The calibration curve (upper right) was established using the following standard proteins: (a) BSA (M_r = 67 000); (b) ovalbumin (M_r = 43 000); (c) chymotrypsinogen A (M_r = 25 000); and (d) ribonuclease A (M_r = 13 700). The protein absorbance (———) and the amylase activity (●, ○, △) were monitored at 280 and 600 nm, respectively. (———) Control serum protein; (●) control serum amylase; (○) case 1 serum amylase; (△) case 2 serum amylase. Peaks 1, 2 and 3 were characterized by the electrophoretic isoenzymic method shown in Fig. 3.

was noted (peak 3) and the corresponding retention time was 40 min (M_r = 12 500).

In case 1, two peaks of amylase activity (peaks 1 and 3) were noted: their retention times were 37 min (M_r 21 000) and 40 min (M_r = 12 500), respectively. In case 2, amylase activity was eluted in a large peak (peak 2). The corresponding retention time was 39 min (M_r = 15 000).

Isoamylase characterization of these peaks was then performed. Electrophoresis on cellulose acetate membranes of the peaks is shown in Fig. 3. From the data obtained, it was confirmed that peak 1 contained fast-migrating abnormal bands of amylase, and that peak 3 corresponded to the bands of normal salivary and pancreatic isoamylase. In case 2, peak 2 (retention time 39 min) contained a small amount (3.5%) of the fast-migrating abnormal bands of amylase. Peak 1 of cases 1 and 2, and peak 3 of the control serum, were then concentrated and used in following experiments.

Neuraminidase treatment and reaction with an anti-salivary amylase monoclonal antibody of the abnormal isoamylases contained in peak 1 of cases 1 and 2

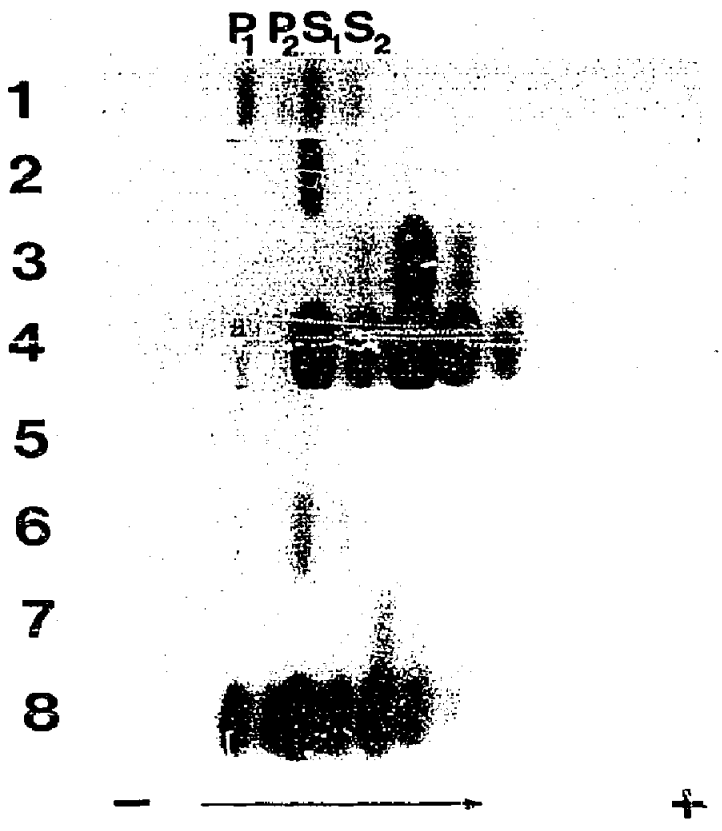

Fig. 3. Electrophoresis on cellulose acetate membrane of peaks 1, 2 and 3 from the HPLC separation shown in Fig. 2. The amylase activity was detected by the blue starch staining technique. 1, Peak 3 of control serum; 2–4, case 1 (2, peak 3; 3, peak 1; 4, original serum); 5–8, case 2 (5, peak 3; 6, peak 2; 7, peak 1; 8, original serum). Fast-migrating isoamylases were observed in peak 1 of each case (lanes 3 and 7).

were carried out. These samples were then submitted to electrophoretic analysis (Fig. 4) and HPLC analysis (Fig. 5). Normal isoamylases contained in peak 3 of control serum were simultaneously submitted to the same analyses. Normal isoamylases showed no change following neuraminidase treatment. On the other hand, the abnormal isoamylases of the both cases showed a reduction of electrophoretic mobility to cathodic side of the abnormal amylases, whose mobility became that of untreated or treated normal salivary isoamylases. The elution peaks of the neuraminidase-treated abnormal isoamylases contained in peak 1 of both cases were equal to the peaks of abnormal isoamylases of original sera, on HPLC analysis (Fig. 5). The elution peak of the neuraminidase-treated normal isoamylases contained in peak 3 of the control serum showed no change either (Fig. 5A).

The amylase activities of the mixture consisting of peak 1 of both cases and the anti-salivary monoclonal antibody were almost completely inhibited (89%). That

of the mixture consisting of peak 3 (P, 42%; S, 58%) of the control serum and the monoclonal antibody was inhibited by 51%. Salivary amylase bands in peak 3 of the control serum disappeared from the electrophoretic pattern following reaction with the monoclonal antibody, and a faint broad band of amylase activity was observed (Fig. 4A). The broad band is characteristic of amylase–immunoglobulin complex formation [11]. The abnormal fast-migrating isoamylase bands of both cases similarly disappeared, and a broad band was formed with the monoclonal antibody (Fig. 4).

The behaviour of these abnormal isoamylases was studied by Con A HPLAC. The abnormal isoamylases of the both cases had no affinity for Con A as purified salivary amylase from whole saliva (data not shown). After immunoblotting of the SDS polyacrylamide gel electropherogram, the molecular mass of the abnormal isoamylases from case 1 was estimated to be *ca.* 60 000, as was that of purified salivary amylase from whole saliva (Fig. 6).

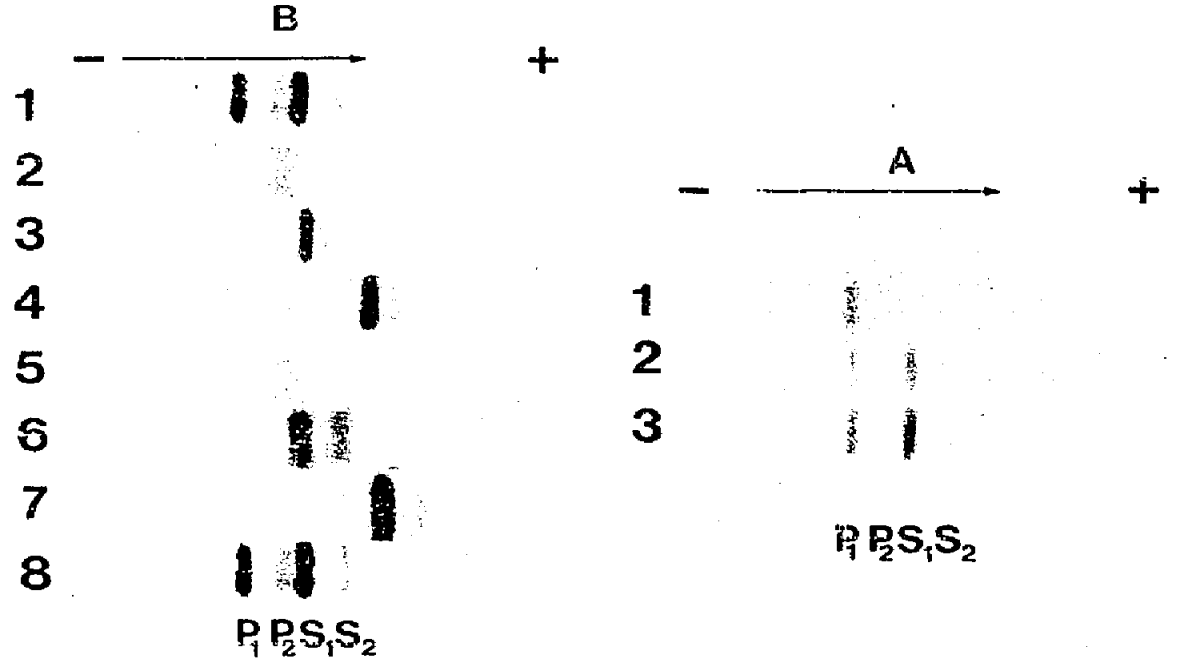

Fig. 4. Electrophoretic isoamylase analyses of peak 1 of patients' sera and peak 3 of control serum, prepared by HPLC separation after neuraminidase treatment and/or reaction with anti-salivary monoclonal antibody. The amylase activity was detected by the blue starch staining technique. (A) Control serum. 1, Peak 3 after reaction with the monoclonal antibody; 2, peak 3 treated with neuraminidase; 3, untreated and/or non-reacted peak 3. (B) Patients' sera. 1 and 8, Untreated and/or non-reacted peak 3 of control serum; 2–4, case 2 (2, peak 1 after reaction with the monoclonal antibody; 3, peak 1 treated with neuraminidase; 4, unreated and/or non-reacted peak 1); 5–7, case 1 (5, peak 1 reacted with the monoclonal antibody; 6, peak 1 treated with neuraminidase; 7, untreated and/or non-reacted peak 1).

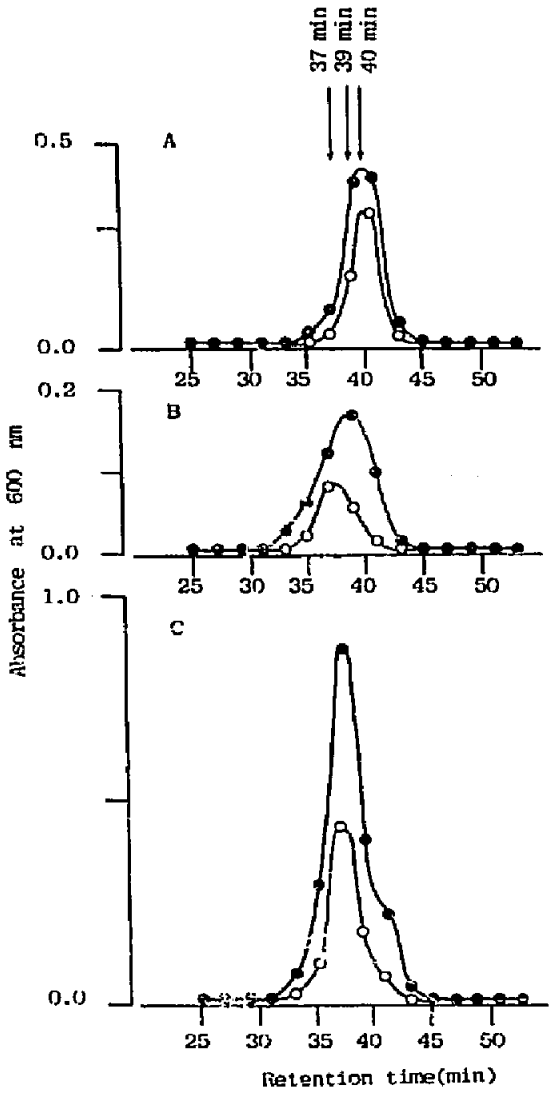

Fig. 5. Superose HPLC elution patterns of neuraminidase-treated abnormal isoamylases contained in peak 1 of Fig. 2; 0.2 ml of the sample solutions was injected separately and eluted at 0.4 ml/min with 150 m*M* sodium chloride–50 m*M* phosphate buffer (pH 7.2). The amylase activity was monitored at 600 nm. (●) Untreated control serum and patient's serum; (○) peak 3 (A) and peak 1 (B, C) treated with neuraminidase. (A) Control serum; (B) case 2; (C) case 1. The peaks containing the abnormal isoamylases, corresponding to a retention time of 37 min, were not changed by the neuraminidase treatment. The peak containing control isoamylases, corresponding to a retention time of 40 min, was not changed either.

DISUCUSSION

Amylase isoenzymes, with an abnormal anodic migration, were detected by an electrophoretic technique in the sera from two patients with IgA-type myeloma. The abnormal isoamylases were not detected in the saliva from case 1. Therefore, the abnormal amylase could be considered as an isoamylase of post-transcriptional modification product. The serum abnormal isoamylases were shown to be

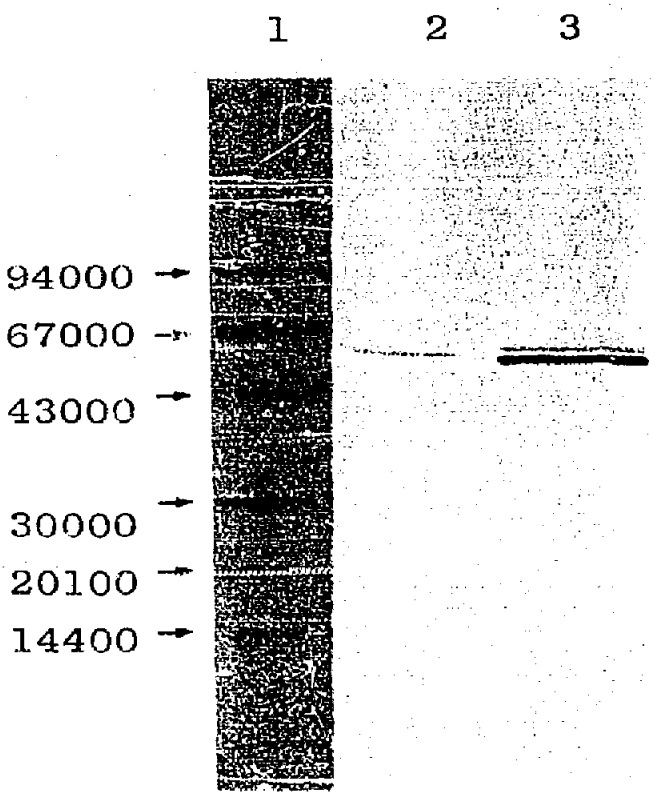

Fig. 6. Immunoblotting patterns of the sialic acid-containing salivary-type amylase prepared from HPLC and purified salivary amylase by SDS-PAGE. 1, Molecular mass markers: phosphorylase b (M_r = 94 000), BSA (M_r = 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r = 30 000), soybean trypsin inhibitor (M_r = 20 100) and alpha-lactalbumin (M_r = 14 400). They were stained with 0.2% Coomassie Brilliant Blue R-250 in 40% methanol–10% acetic acid. 2, Peak 1 of case 1 from HPLC separation. 3, Purified salivary amylase from human whole saliva.

sialic acid residues containing amylase from a study of treatment with neuraminidase, and to be salivary-type amylase after a study of reaction with anti-human salivary monoclonal antibody. The sialic acid-containing salivary-type amylases could be separated clearly from residual normal isoamylases of patients' sera by size-exclusion HPLC on a Superose 12 column. Although amylase activity was eluted into a large peak in case 2, the higher content of normal isoamylases meant that the abnormal amylase could be separated.

The abnormal salivary-type amylases were then studied, in comparison with purified salivary amylase, by Con A HPLAC and SDS-polyacrylamide gel electrophoris (PAGE). As a result, the abnormal amylase had no affinity for Con A, and its molecular mass was estimated to be *ca.* 60 000 as purified salivary amylase. This value is close to that reported by Takeuchi [16].

There is a large discrepancy between the molecular mass values determined for serum amylases by gel permeation HPLC (M_r = 12 500–20 000) and that of 60 000 determined by SDS-PAGE. During dextran gel permeation chromatography, it was observed that the elution of amylase molecules was considerably retarded, because dextran has a chemical structure similar to the substrate of amylase [21]. Interaction between agarose of Superose 12 column matrix and amylase molecules has not been previously reported. However, similar retardation of amylase in the Superose 12 column was noted during our HPLC analyses.

On the other hand, although the molecular mass of the sialic acid-containing salivary-type amylase equals that of the purified salivary amylase, the former amylase was well separated by HPLC analysis on a Superose 12 column. Moreover, the peak of the abnormal salivary-type amylase did not change following neuraminidase treatment. We consider that such elution behaviour can be explained by the unusual protein conformation of this abnormal salivary-type amylase.

This is the first reported study in which the phenotype for sialic acid-containing amylase has been elucidated immunologically to be salivary-type and in which this amylase has been detected in patients' sera with myeloma. Sudo and Kanno found sialic acid-containing amylase in pancreatic cancer [10] and lung cancer [22], respectively. Nakayama *et al.* [3] also reported that this amylase is found in lung cancer. Sandiford and Chiknas [23] found fast-migrating amylase in the serum of a patient with advanced ovarian cancer but its properties were not studied. The latter amylase [23] can be presumed, from our study, to be sialic acid-containing salivary-type amylase. However, acidic amylase from ovarian cystic fluids [24,25] and lymphoepitherial cyst [26] can be distinguished clearly from the sialic acid-containing salivary type amylase. These cystic amylases are unaffected by treatment with neuraminidase. Therefore, these amylases are thought to result from ageing transformation of cystic amylase, as reported by Warshaw and Lee [27] and Weaver *et al.* [28].

There remains yet one question concerning the present two cases. Where were these unique isoamylases produced? In 1983, Zakrzewsha and Prokopowicz [29] 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 leukocytic maturation. The phenotype of amylase in leukocytes is salivary-type, as reported by Vacikova [30]. In 1988, Hata *et al.* [9] demonstrated that salivary-type amylase was produced by human myeloma cells in a patient with IgA-type myeloma.

We are researching the origin of the sialic acid-containing salivary-type amylases on the assumption that this amylase is produced ectopically by myeloma cells, although there is as yet no direct evidence of this in our two patients.

There have been several recent immunohistochemical studies on human amylase tissues, particularly in the lung and female genital tract [31] and the fallopian tube [32]. Therefore, the production of amylase by tumours is easily understood

as the activation and exaggeration of amylase contained in the normal tissues rather than as ectopic production. We propose that the ectopic production of salivary-type amylase myeloma cells [9] is an analogous phenomenon to other amylase-producing tumours. Accordingly, if amylase isoenzyme electrophoresis is more widely applied to hyperamylasemia, we think that many cases of amylase-production patients with myeloma and/or monoclonal gammapathy will be reported in the future.

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