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LIQUID CHROMATOGRAPHIC ASSAY OF THE RELATIVE ACTIVITIES OF SERUM PANCREATIC AND SALIVARY α-AMYLASE USING REDUCTIVELY PYRIDYLAMINATED MALTOPENTAOSE AS A FLUORESCENT SUBSTRATE

SUSUMU HONDA*, TAKESHI OHMURA, YOKO SEGAWA, TOSHIYE MINEMATSU and KAZUAKI KAKEHI

Faculty of Pharmaceutical Sciences, Kinki University, Kowakae, Higashi-Osaka 577 (Japan)

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SUMMARY

A method for the high-performance liquid chromatographic assay of the relative activities of serum pancreatic and salivary α-amylase has been developed, using maltopentaose reductively aminated with 2-aminopyridine as a fluorescent substrate. Both enzymes showed similar modes of action, cleaving the second and the third (from the non-reducing terminal) interglycosidic linkages of this substrate. However, the relative ease of cleavage of these two sites by the pancreatic enzyme was significantly different from that by the salivary enzyme. Therefore, determination of the molar ratio of the cleavage products by HPLC could lead to estimation of the activity ratio of these enzymes. The optimum chromatographic conditions for HPLC were as follows: column, LiChrosorb RP-18 (Merck, 7 μm, 250 $\times$ 4 mm I.D.); column temperature, ambient; eluent, 0.01% orthophosphoric acid–acetonitrile (4:1, v/v) containing 2.4 m*M* sodium laurylsulphate; flow-rate, 1.0 ml/min; wavelengths for fluorimetric detection, 320 nm (excitation)/400 nm (emission). The problem of interference by serum α-glucosidase was solved by specific inhibition with tris(hydroxymethyl)aminomethane and erythritol. The data obtained by the proposed method correlated well with those produced by the conventional method based on electrophoresis.

INTRODUCTION

For accurate diagnosis of some pancreatic disorders it is important to estimate individual activities (or their ratio) of serum pancreatic and salivary α-amylases besides the total activities. For example, it is known that in chronic pancreatitis with exocrine insufficiency the activity of pancreatic α-amylase in serum is decreased, whereas total α-amylase activities remain at the normal level [1]. Owing to the close resemblance of substrate specificity, these enzymes are usually discriminated from each other by direct separation using electrophoresis on cel-

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lulose acetate [2] or electrofocusing [3]. Immunological methods [4] are also available, though not so practical. However, all these methods are time-consuming and have low reproducibility. Recently, an approach was made to differentiate these enzymes, based on their different inhibitory effects on wheat flour proteins [5,6]. On the other hand, Ohmichi and Ikenaka [7] developed a method based on high-performance liquid chromatography (HPLC) of the digestion products from a fluorescent substrate, O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4‴)-maltotetraose, i.e. maltopentaose with a fluorescent label at the non-reducing terminal. The modes of action of pancreatic and salivary α-amylases on this substrate are sufficiently different for these enzymes to be differentiated. In addition, the method is highly sensitive. Nevertheless, preparation of this substrate is rather tedious, because it involves chemical oxidation of the primary hydroxyl groups in maltodextrins followed by fluorescent labelling and partial enzymatic hydrolysis. The product should be resolved chromatographically from accompanying isomers and oligosaccharides with different degree of polymerization, and its yield is low.

In this paper we propose the use of an analogous substrate, N-2-pyridyl-[1-deoxy-(4-O-α-maltotetraosyl)-D-glucitol-1-yl]amine (G5-AP), i.e. maltopentaose with a fluorescent label at the reducing end, which is more easily prepared and less expensive. Although serum α-glucosidase interfered with the determination of α-amylases by concurrent cleavage of the glycosidic linkages in the substrate, it was masked by the use of a selective inhibitor. We demonstrate the usefulness of this improved method for the assay of the ratio of pancreatic and salivary α-amylase activities.

EXPERIMENTAL

Materials

A sample of human salivary α-amylase was obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification. A sample of human pancreatic α-amylase was a gift from Dr. Kurooka. Both enzymes gave single bands when examined by electrophoresis on a cellulose acetate membrane according to the procedure of Davis [8]. Each sample of α-amylases was diluted with 20 m*M* phosphate buffer (pH 6.9) containing 150 m*M* sodium chloride, and the diluted solutions were immediately used. Maltooligosaccharides were purchased from Wako (Osaka, Japan). 4-Methylumbelliferyl α-D-glucoside was obtained from Seikagaku Kogyo (Tokyo, Japan). 2-Aminopyridine and sodium cyanoborohydride were from Nakarai (Kyoto, Japan). Other reagents and solvents were of the highest grade commercially available. The control serum (Precinorm®U) as a source of α-glucosidase was obtained from Böhringer (Mannheim, F.R.G.). Serum samples were collected from volunteers aged 22–50 years. Bondelut® columns for cleanup of digestion mixtures were obtained from Analytical International (Harbor City, CA, U.S.A.).

Instrumentation

The HPLC apparatus of a Hitachi 638 high-performance liquid chromatograph, a Rheodyne injector with a 20-μl loop, a Hitachi 650-10-LC spectrofluo-

TABLE I

YIELDS AND ABBREVIATIONS OF REDUCTIVELY PYRIDYLAMINATED MALTOOLIGOSACCHARIDES

Starting material	Yield of the refined product (%)	Abbreviation
Glucose	84	G1-AP
Maltose	77	G2-AP
Maltotriose	73	G3-AP
Maltotetraose	73	G4-AP
Maltopentaose	72	G5-AP
Maltohexaose	75	G6-AP
Maltoheptaose	72	G7-AP

romonitor equipped with a 90-μl quartz cell, and a Schimadzu Chromatopak E1-A integrator. A Hibar LiChroCART column (250×4 mm I.D.) packed with LiChrosorb RP-18 (7 μm) was purchased from Merck (Darmstadt, F.R.G.). Elution was carried out with 0.01% orthophosphoric acid–acetonitrile (4:1, v/v) containing 2.4 m*M* sodium laurylsulphate at a flow-rate of 1.0 ml/min. The wavelengths for fluorimetric monitoring were 320 nm (excitation) and 400 nm (emission). A search for an inhibitor of α-glucosidase was made in the flow-injection mode by using the above apparatus without the analytical column.

Preparation of reductively pyridylaminated maltooligosaccharides

These compounds were prepared by treating maltooligosaccharides with 2-aminopyridine in methanol in the presence of sodium cyanoborohydride and acetic acid, according to the procedure of Hase et al. [9]. Structures of the products were confirmed by ^{1}H NMR and fast atom bombardment mass spectra. The spectral data will be presented elsewhere, together with those of related compounds. The yields and the abbreviations of the products are listed in Table I.

Procedure for enzyme reaction

Diluted enzyme solution (5 μl) was added to 20 m*M* phosphate buffer (pH 6.9, 180 μl) containing 6 m*M* sodium chloride. The reaction was started at 37°C by adding an aqueous 5 m*M* solution (40 μl) of G5-AP, and continued for 20 min. The mixture was heated for 3 min on a boiling water-bath to inactivate the enzyme, then diluted with the HPLC eluent (500 μl). An aliquot (20 μl) of the solution was analysed by HPLC. The amounts of G2-AP and G3-AP produced were estimated, together with that of remaining G5-AP, from their peak heights relative to those obtained with a standard mixture of G2-AP, G3-AP and G5-AP.

Survey for a selective inhibitor of serum α-glucosidase

A 2.2 m*M* solution (20 μl) of 4-methylumbelliferyl α-D-glucoside in 0.1 *M* acetate buffer (pH 4.0) was mixed with a 22 m*M* solution (20 μl) of one of the compounds examined for inhibitory effect in the same buffer. A sample of control

serum (10 μl) as a source of α-glucosidase was added to the mixture, and the mixture was incubated for 30 min at 37°C. Subsequently, 0.5 *M* carbonate buffer (pH 10.7, 500 μl) was added to stop the reaction, and an aliquot (10 μl) was injected into a stream of carrier (0.5 *M* carbonate buffer, pH 10.7) flowing at a rate of 1.0 ml/min, led into the fluoromonitor. In parallel with this experiment a blank test was performed in which the solution of the compound examined for inhibitory effect was replaced by water. From the ratio of the decrease of fluorescence intensity at 365 nm (excitation)/448 nm (emission) to that of the intensity of the blank, the inhibition percentage was obtained.

Estimation of the ratio of the activities of pancreatic and salivary α-amylases in sera

To 20 m*M* phosphate buffer (pH 6.9, 100 μl) containing 6 m*M* sodium chloride, were added 5 m*M* G5-AP (40 μl) and an aqueous solution (80 μl) containing tris(hydroxymethyl)aminomethane and erythritol at an equal concentration of 110 m*M*. The reaction was started at 37°C by adding a serum sample (50 μl) to the mixture, and continued for 20 min. The reaction was stopped by heating the mixture for 3 min at 100°C. The HPLC eluent (300 μl) was added to the reaction mixture, and the mixture was centrifuged at 1700 *g* to remove proteinaceous substances. The supernatant was then passed through a Bondelut column containing octadecyl-silica (1 ml), and the column was washed with aqueous 50% methanol (5 ml). The combined eluate and the washing fluids were evaporated to dryness, and the residue was dissolved in the eluent (300 μl). An aliquot of the solution (20 μl) was analysed by HPLC.

RESULTS AND DISCUSSION

Separation of reductively pyridylaminated maltooligosaccharides

All reductively pyridylaminated maltooligosaccharides contain an imino and a pyridyl group. Because of their cationic and hydrophobic nature, their mixtures could be separated as ion-pairs with laurylsulphate on an octadecyl-silica gel column. Fig. 1 shows a typical chromatogram obtained from a mixture of G*n*-APs, where *n* ranges from 1 to 7 (see abbreviations in Table I). All these derivatives were completely separated from each other within 60 min and sensitively detected by fluorescence, under the conditions described in Experimental.

Selection of an appropriate substrate for the assay of the proportion of pancreatic and salivary α-amylase activities

Fig. 2 shows elution profiles of the reaction products from reductively pyridylaminated maltooligosaccharides with different degree of polymerization, produced by the action of pancreatic and salivary α-amylases. Compound G7-AP (rank A) was hydrolysed by both α-amylases to give G3-AP and G4-AP. The G3-AP to G4-AP peak-height ratio was, however, almost the same. Action of either enzyme on G6-AP (rank B) gave G3-AP as the only product. A small peak of G7-AP was also observed in the chromatogram, which presumably arose by transglycosidation. On the other hand, the modes of action of these α-amylases

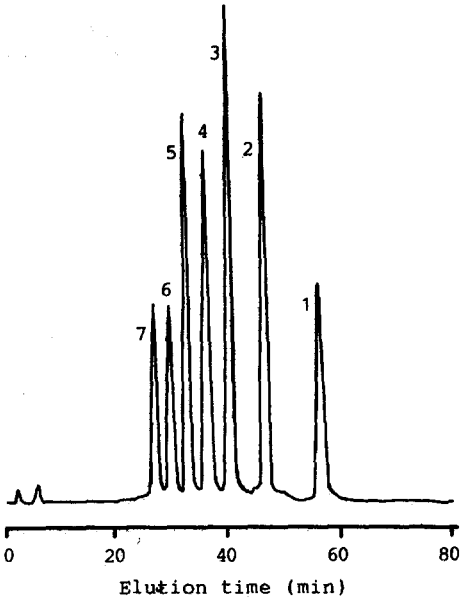

Fig. 1. Separation of reductively pyridylaminated maltooligosaccharides with different degrees of polymerization. Column, Hibar LiChroCART (250×4 mm I.D.) packed with LiChrosorb RP-18 (7 μm); column temperature, ambient; eluent, 0.01% phosphoric acid-acetonitrile (4:1, v/v) containing 2.4 m*M* sodium laurylsulphate; flow-rate, 1.0 ml/min; wavelengths for detection, 320 nm (excitation)/400 nm (emission). Peaks: 1 = G1-AP; 2 = G2-AP; 3 = G3-AP; 4 = G4-AP; 5 = G5-AP; 6 = G6-AP; 7 = G7-AP. Amounts injected: 1, 210 pmol; 2, 240 pmol; 3, 240 pmol; 4, 180 pmol; 5, 210 pmol; 6, 120 pmol; 7, 120 pmol.

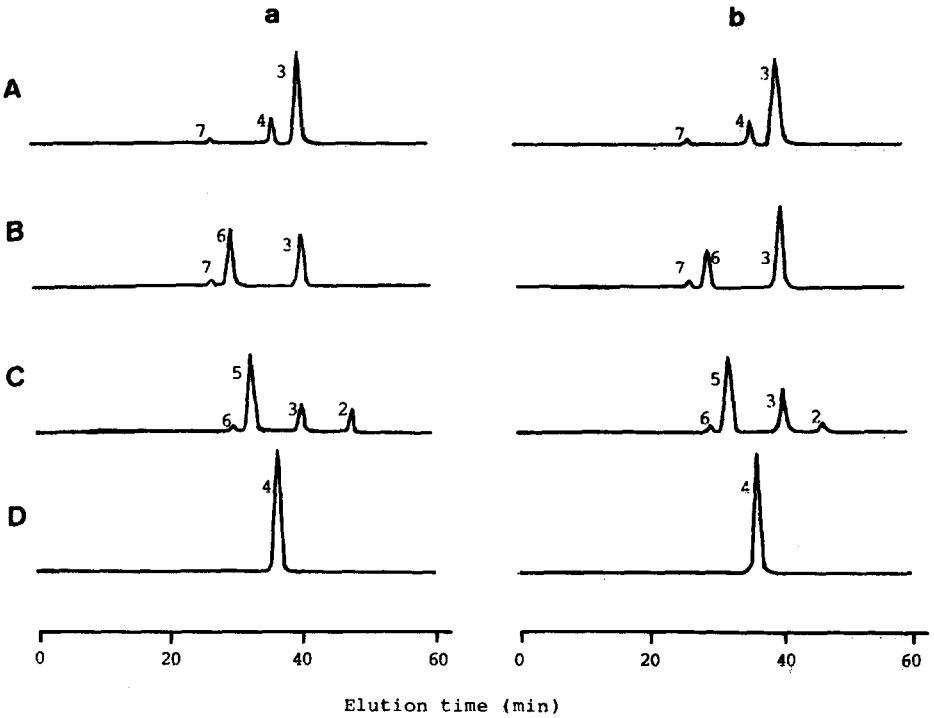

Fig. 2. Comparison of cleavage patterns of reductively pyridylaminated maltooligosaccharides. Analytical conditions as in Fig. 1. (a) Pancreatic α-amylase; (b) salivary α-amylase. Substrates: (A)

on G5-AP (rank C) were significantly different. Although both α-amylases gave G3-AP and G2-AP, the product ratio was sufficiently different to allow differentiation of the enzymes via the peak-height ratio. Neither α-amylase cleaved G4-AP, the peak of the raw material being detected in rank D as the only peak. The same result was obtained for G3-AP and G2-AP, though chromatograms are not shown.

Calibration curves

The calibrations curves of G2-AP and G3-AP derivable from G5-AP were linear in the range 50–600 pmol under the aforementioned conditions. G5-AP itself also gave a straight line in the same range. The lower limit of detection of these compounds was commonly 3 pmol.

Reproducibility

Repeated determinations ($n=10$) of G2-AP and G3-AP at the 60 pmol level indicated that the coefficient of variation (C.V.) was 2.7% and 2.2%, respectively. The C.V. of the retention time was 1.8% and 1.7%, respectively. These data indicate that the proposed HPLC method was sufficiently reproducible for routine analysis of these derivatives.

On the basis of these observations, G5-AP was adopted as a substrate for the assay of the ratio of pancreatic and salivary α-amylase activities.

Interference by α-glucosidase

It has been shown that an exo-type α-glucosidase (E.C. 3.2.1.20) is present in several tissues and body fluids [10], hence the methods for α-amylase assay utilizing 1,4-α-glucans and their derivatives as substrates suffer from interference by this enzyme. In the present method a part of the substrate (G5-AP) was also hydrolysed at the outermost interglycosidic linkage to give G4-AP, as evidenced by the result obtained when G5-AP was incubated with control serum known to contain this α-glucosidase (Fig. 3c). This chromatogram was quite different from those obtained by digestion with authentic pancreatic (Fig. 3a) and salivary (Fig. 3b) α-amylases. A part of the G4-AP produced by the action of α-glucosidase should have been further degraded successively to G3-AP and G2-AP. Therefore, peaks 3 and 2 in Fig. 3c were considered to be of the overall G3-AP and G2-AP, respectively, produced by the combined action of α-amylases and α-glucosidase.

Since the interference by α-glucosidase could not be ignored, we searched for a specific inhibitor of α-glucosidase. We examined more than 30 compounds, and found some to be effective. The inhibition for selected compounds were as follows: maltitol, 14%; ampicillin, 19%; kanamycin, 21%; turanose, 26%; *p*-nitrophenyl α-D-glucoside, 53%; maltose, 69%; maltotriitol, 82%; acarbose, 97%; an equimolar mixture of tris(hydroxymethyl)aminomethane and erythritol, 97%, when 44 μmol was added to a 20-μl sample of control serum as a source of α-glucosidase. The inhibitory effects of acarbose and an equimolar mixture of tris(hydroxymethyl)aminomethane and erythritol is remarkably strong (97% in both cases). The latter has previously been reported by Jorgensen and Jorgensen [11], and our present data support their finding. It is significant that this com-

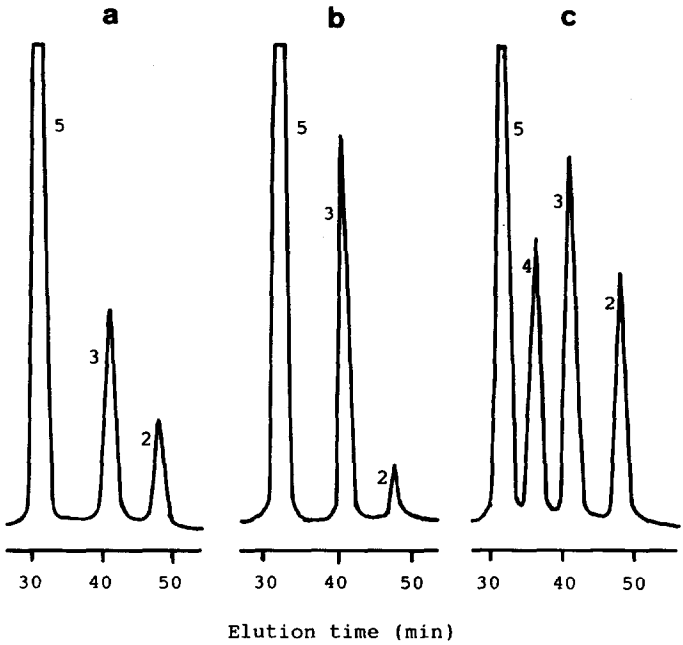

Fig. 3. Comparison of cleavage patterns of G5-AP: (a) pancreatic α-amylase; (b) salivary α-amylase; (c) control serum (source of α-glucosidase). Analytical conditions and peak numbers as in Fig. 1.

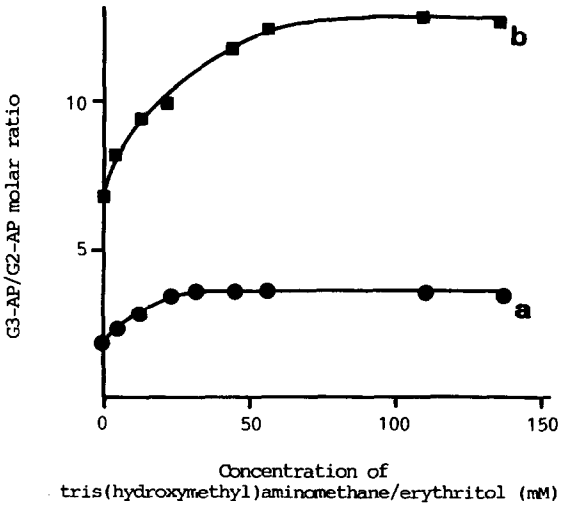

Fig. 4. Effect of the concentration of tris(hydroxymethyl)aminomethane-erythritol on the molar ratio of G3-AP to G2-AP. Curve a, pancreatic α-amylase; curve b, salivary α-amylase. The concentrations of these two compounds were equimolar for all plots.

bination showed little inhibitory effect on both α-amylase activities. In contrast, acarbose inhibited both α-amylase activities almost completely, in addition to α-glucosidase activity. The addition of the tris(hydroxymethyl)aminometh-

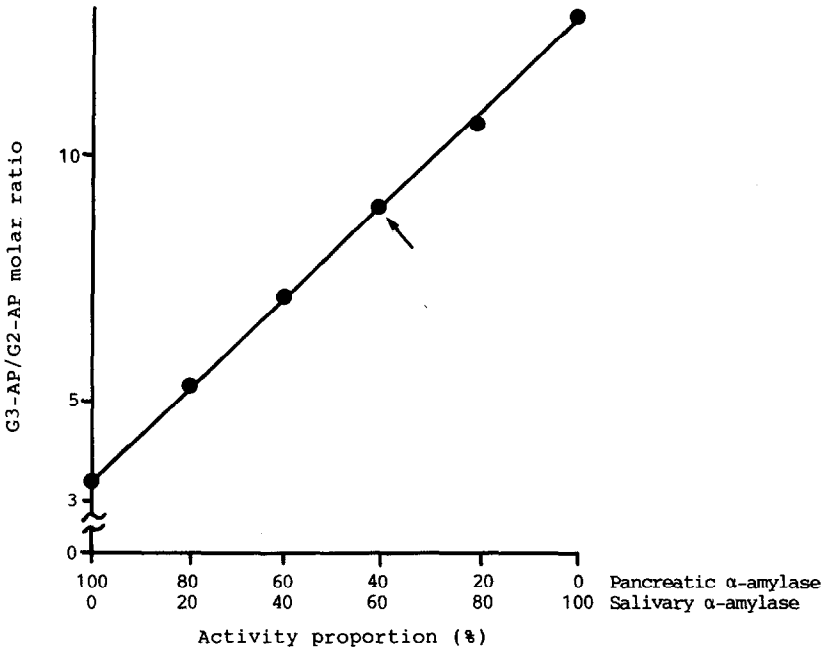

Fig. 5. Relationship between the molar ratio of G3-AP to G2-AP and the proportions of pancreatic and salivary α-amylase activities. The regression line is $y=0.94\,x+3.44$ ($r=0.999$). At the point of the arrow, the C.V. ($n=6$) was 4.0%.

ane–erythritol did not interfere with the analysis of G2-AP and G3-AP, but altered the molar ratio of G3-AP to G2-AP. For concentrations of this combination below 50 m*M*, the molar ratio was gradually increased for both α-amylases, indicating incomplete inhibition (Fig. 4). At 50 m*M* plateaux were reached for both α-amylases, indicative of completion of inhibition. We chose a concentration of 110 m*M* for the present study. This choise is arbitrary because little information was available with regard to the serum α-glucosidase level; however, it is considered to be at least sufficiently high to allow α-amylase assay for sera containing normal levels of α-glucosidase. Under these conditions, the molar ratio of G3-AP to G2-AP for pancreatic and salivary α-amylases was 3.4 and 12.8, respectively.

Estimation of the ratio of pancreatic and salivary α-amylase activities

The relationship between the G3-AP to G2-AP molar ratio and the proportions of pancreatic and salivary α-amylase activities was linear over the whole range of activities investigated (Fig. 5). Therefore, the relative activities of these two α-amylases could be easily estimated from the molar ratio of G3-AP to G2-AP. The C.V. ($n=6$) at the point of 40% of pancreatic α-amylase activity (indicated by the arrow) was 4.0%.

Correlation to the electrophoretic method

The pancreatic to salivary α-amylase activity ratio was estimated for a number of serum samples by the proposed method and the conventional method based on

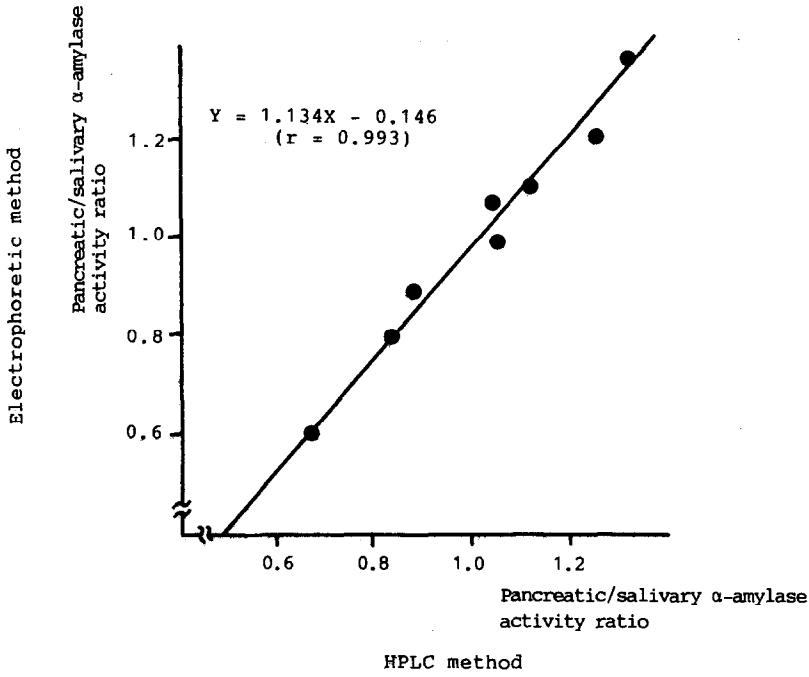

Fig. 6. Correlation of the data for the ratio of the activities of pancreatic and salivary α-amylases obtained by the proposed method with those obtained by the conventional electrophoretic method.

electrophoresis [8]. Good correlation was observed between the data obtained by these two methods (Fig. 6). The correlation coefficient was very close to unity (0.993). Since the electrophoretic method involves a staining step, which is based on a heterogeneous reaction between the enzyme and the insoluble substrate, reproducibility requires dexterity. In contrast, our proposed method was highly reproducible and did not require any special techniques. Shorter analysis time is also an advantage of this method.

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