

# Electrophoretic Determination of Isoamylases in Serum with Commercially Available Reagents

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We describe and evaluate a method for determining isoamylases in serum. With this method, which involves conventional electrophoretic apparatus and commercially available reagents, seven isoenzymes can be resolved. The electrophoresis is performed in agarose gel, and isoenzymes are detected with a water-insoluble cross-linked starch polymer linked to a blue dye. The isoenzymes are quantified by densitometry. Within-assay imprecision (CV) was <7%, between-assay CV <11%. This method has the sensitivity to detect the major isoamylases (P<sub>2</sub> and S<sub>2</sub>) in the serum, even at low total amylase activity. Reference intervals for P-type isoenzymes were 2-52 U/L and for S-type 2-85 U/L. P<sub>2</sub>S<sub>2</sub> is the most common pattern found in normal individuals, then (in decreasing order) P<sub>1</sub>P<sub>2</sub>S<sub>2</sub>, P<sub>2</sub>S<sub>2</sub>S<sub>3</sub>, and P<sub>2</sub> patterns. Patterns found in serum from cases of acute pancreatitis, mumps, macroamylasemia, and pelvic inflammatory disease are illustrated.

**Additional Keyphrases:** *electrophoresis, agarose gel · P- and S-type amylase isoenzymes · reference interval · pancreatitis · mumps · macroamylasemia · methods for the small laboratory · enzyme activity*

Serum amylase ( $\alpha$ -1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) activity has been reported to be increased not only in acute pancreatitis but also in many other pathological conditions (1-3). Separation of amylase isoenzymes is useful in the differential diagnosis of conditions responsible for hyperamylasemia (2-9). Different methods, including chromatography, electrophoresis, and isoelectric focusing, have been proposed for doing this (5-10), but most of them are not readily applicable to routine use in a clinical laboratory.

In this paper we describe an agarose gel electrophoretic method with commercially available reagents, including agarose plates and a simple electrophoretic apparatus. This method, a modification of that described by Skude (11), can be easily used in most clinical laboratories.

## Materials and Methods

### Materials

**Apparatus:** We used a Model 310 electrophoresis cell and power supply (G. K. Turner Associates, Palo Alto, CA 94303) and quantified the isoamylases with an electrophoresis densitometer (Model 345; Clifford Instruments, Inc., Natick, MA 01760). Total amylase activity was determined with a Multistat III (Instrumentation Laboratory, Lexington, MA 02173).

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**Reagents:** The agarose plates (Special Purpose Electrophoresis Film®, cat. no. 470104; Corning Medical, Palo Alto, CA 94306) contained, per liter, 10 g of agarose, and 50 g of sucrose in 2-amino-2-methyl-1-propanol buffer, 30 mmol/L, pH 8.6. The electrophoresis buffer, prepared from the dry Special Purpose Barbitol Buffer from Corning, contained sodium barbital, 50 mmol/L, pH 8.6. Phadebas® Amylase Test tablets were obtained from Pharmacia Diagnostics, Uppsala, Sweden. The visualization buffer was a 0.1 mol/L phosphate buffer (pH 7.0) that contained, per liter, 5 g of human serum albumin (Cutter Laboratories, Inc., Berkeley, CA 94710), 8.5 g of sodium chloride, and 10 g of potassium chloride. The rinsing solution was a mixture of methanol/acetic acid/water (5/1/5, by vol). Reagents for total amylase activity measurement were obtained through BMC Canada, Dorval, Canada, H9P 1A9.

### Procedures

**Amylase total activity:** Amylase activity was measured by centrifugal analysis at 30 °C with BMC's "Single Vial" amylase kit, in which maltotetraose is the substrate for amylase. One unit (U) of amylase initiates a series of reactions that lead to the reduction of 1  $\mu$ mol of NAD<sup>+</sup> per minute.

**Electrophoresis:** Table 1 specifies the sample volume to be applied in the well of the agarose film. Dilutions are made with sodium chloride solution (8.5 g/L). Keep the electrophoresis buffer cold (4 °C), and use a current of 11 mA (dc) for 75 to 150 min, depending on the degree of separation desired. For routine analysis, 120 min is preferred.

**Visualization and quantification of amylase isoenzymes:** Suspend seven tablets of Phadebas Amylase Test (a cross-linked potato-starch polymer carrying a blue dye) in 10 mL of the visualization buffer and pour the mixture uniformly over the gel. Incubate the plate for 2 h at 56 °C in a moist chamber, then wash the plate with distilled water and soak in the rinsing solution for 1 h. Air-dry the gel in an oven set at 30 °C. Quantitate the isoenzymes with a densitometer, using the 540-nm filter.

**Preparation of salivary and pancreatic extracts:** Centri-

**Table 1. Sample Volume Applied for Electrophoresis, Depending on Total Amylase Activity**

Amylase activity, U/L ( $\times$ URL <sup>a</sup> )	Sample vol applied, $\mu$ L
<15 (<0.13)	4.8
15-30 (0.13-0.26)	3.2
30-100 (0.26-0.87)	1.6
>100 (>0.87)	1.6 after diln to 70 U/L (0.61 $\times$ URL <sup>a</sup> )

<sup>a</sup> Upper reference limit (URL), 115 U/L.

fuge saliva and dilute the supernate with sodium chloride solution (8.5 g/L) to an appropriate amylase activity.

Obtain normal pancreas tissue at autopsy, homogenized in saline, and centrifuge; dilute the supernate as for saliva.

### Evaluation of the Method

**Precision:** We prepared two controls: the activity in control A was about 80% pancreatic amylase (from the pancreatic extract) and 20% salivary amylase (from the salivary extract); that in control B was 20% pancreatic amylase and 80% salivary amylase. The total activity in each was about 120 U/L. We evaluated within-assay precision for each isoenzyme by measuring each control 19 times, in duplicate. Between-assay precision was obtained by making 22 consecutive assays on the two controls.

**Sensitivity:** We evaluated the isoenzyme pattern of individuals whose total amylase activity in serum was below the lower reference limit (<5 U/L).

**Linearity and activity of isoenzymes for each substrate:** We prepared an enzyme mixture of which about half was amylase activity from pancreatic extract and half was activity from salivary extract as measured with the BMC method. We diluted this mixture so as to have three samples with respective total amylase activities of 104, 52, and 26 U/L. Each fraction was separated by electrophoresis and reacted with Phadebas substrate; the percentage accounted for was evaluated by densitometry.

### Reference Interval for Isoamylases

The isoenzyme patterns and activities were evaluated in sera from 66 ostensibly healthy fasting subjects, 34 women and 32 men, ages 25 to 45 years. Reference values were established after logarithmic transformation as the mean  $\pm 2.22$  SD, where 2.22 is a factor to include 95% of the population with 0.90 confidence when the population sample size is 70 (12).

## Results and Discussion

### Nomenclature of Amylase Isoenzymes

We have designated in this paper the isoenzymes of amylase in the same manner as that described by Hobbs and Aw (13). These are, from cathode to anode: P<sub>1</sub>, P<sub>2</sub>, P<sub>3</sub>, S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, and S<sub>4</sub>. P<sub>1</sub>, P<sub>2</sub>, and P<sub>3</sub> are named collectively "P (pancreatic) type" isoenzymes and S<sub>1</sub>, S<sub>2</sub>, S<sub>3</sub>, and S<sub>4</sub>, "S (salivary) type" isoenzymes.

The P-type isoenzymes are considered specific to the pancreas (2, 5). P<sub>1</sub> was found in pancreatic homogenate by Magid et al., who called it "fraction 5" (14), and by Skude (11). We

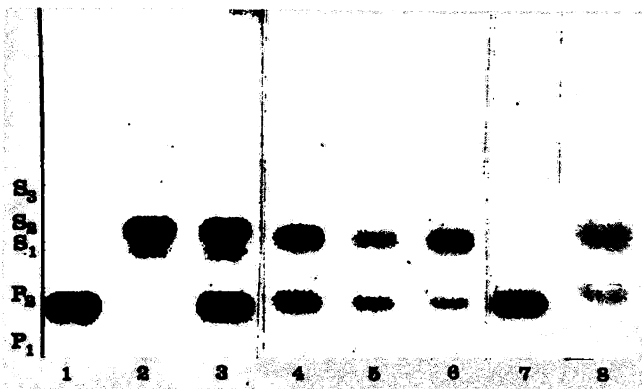


Fig. 1. Electrophoretograms of amylase isoenzymes

Anode at top, origin slightly above P<sub>2</sub> isoenzyme. 1, pancreatic extract (P<sub>2</sub>). 2, salivary extract (S<sub>1</sub>S<sub>2</sub>S<sub>3</sub>). 3, a mixture of salivary and pancreatic extracts (P<sub>2</sub>S<sub>1</sub>S<sub>2</sub>S<sub>3</sub>). 4-7, patterns found in sera from normal individuals: P<sub>2</sub>S<sub>2</sub>, P<sub>1</sub>P<sub>2</sub>S<sub>2</sub>, P<sub>2</sub>S<sub>2</sub>S<sub>3</sub>, and P<sub>2</sub> respectively. 8, P<sub>2</sub>S<sub>2</sub> pattern found in serum with low total amylase activity (5 U/L)

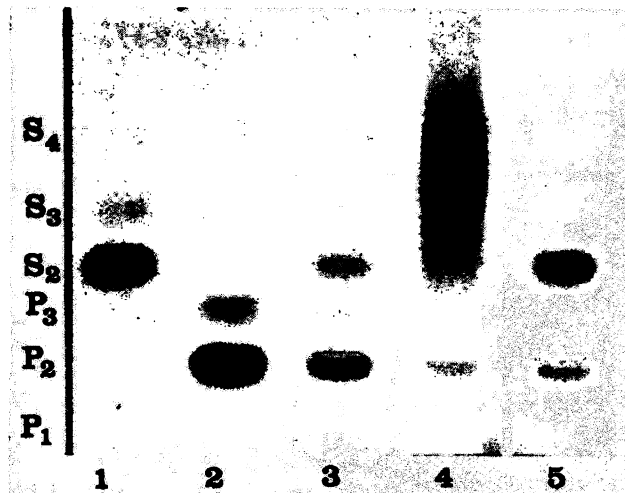


Fig. 2. Electrophoretograms of amylase isoenzymes in serum from various pathological conditions

Anode at top, origin above P<sub>2</sub> isoenzyme. 1, mumps (S<sub>2</sub>S<sub>3</sub>). 2, acute pancreatitis, total amylase high (P<sub>2</sub>P<sub>3</sub>). 3, acute pancreatitis, total amylase normal (P<sub>2</sub>P<sub>3</sub>S<sub>2</sub>). 4, macroamylasemia. 5, pelvic inflammatory disease (P<sub>2</sub>S<sub>2</sub>S<sub>3</sub>S<sub>4</sub>)

also found high P<sub>1</sub> activity in the serum of one patient with acute pancreatitis. P<sub>2</sub> was found in pancreatic extract (Figure 1) and its activity was increased in the serum during acute pancreatitis (Figure 2), as reported by others (11, 14, 15). P<sub>3</sub> was detected in sera from patients with acute pancreatitis (Figure 2) (7, 11), in pancreatic cyst by Magid et al. ("fraction 3") (14), and in pancreatic secretions (7).

We found S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub> (Figure 1) in salivary extracts and S<sub>4</sub> in the serum of a patient with mumps. Hobbs and Aw (13) and Benjamin and Kenny (15) found S<sub>1</sub> to be the major S-type isoenzyme and did not describe the fourth isoenzyme, S<sub>4</sub>. With our method, S<sub>2</sub> was found to be the major S-type isoenzyme. For this reason, we think that our S<sub>2</sub> is the same isoenzyme as the S<sub>1</sub> isoenzyme described by these authors and that S<sub>3</sub> and S<sub>4</sub> are equivalent to their S<sub>2</sub> and S<sub>3</sub>, respectively. S<sub>1</sub> is a slow anodally migrating S-type isoenzyme not resolved by them. We detected S<sub>1</sub> only in salivary extracts, but Skude (11) reported a similar fraction in sera with high salivary-type amylase activity.

### Evaluation of the Method

**Precision:** The within-assay and between-assay precision (Table 2) compares favorably with the results published by others (14, 16).

**Sample stability:** During our between-assay precision study, a sample was stored at -20 °C for three months and frozen and thawed 20 times. We compared the mean total amylase activity and the percentage of P- and S-types from five measurements at the beginning and at the end of this

Table 2. Assay Precision

Isoenzymes type:	Control A		Control B	
	P	S	P	S
<i>Within-assay</i>				
Mean % of total activity <sup>a</sup>	79.9	20.1	18.6	81.4
CV, %	1.6	6.9	4.5	1.1
<i>Between-assay</i>				
Mean % of total activity <sup>a</sup>	80.0	20.0	18.8	81.2
CV, %	2.8	11.1	9.3	2.5

<sup>a</sup> Total activity for each control was about 120 U/L.

**Table 3. Linearity and Activity of Isoenzymes for Each Substrate**

Total amylase activity, U/L <sup>a</sup>	P-type activity recovered, <sup>b</sup>	S-type activity recovered, <sup>b</sup>	Signif. <sup>d</sup>
	U/L ± SD (%) <sup>c</sup>	U/L ± SD (%) <sup>c</sup>	
26	16 ± 0.16 (61.5)	10 ± 0.16 (38.5)	<i>p</i> < 0.001
52	30 ± 1.71 (57.7)	22 ± 1.71 (42.3)	<i>p</i> < 0.01
104	56 ± 1.04 (53.8)	48 ± 1.04 (46.2)	<i>p</i> < 0.01

<sup>a</sup> Total amylase activity measured with maltotetraose as substrate; 50% of pancreatic origin and 50% of salivary origin. <sup>b</sup> Mean activity of P-type and S-type isoenzymes recovered after electrophoresis and reaction with Phadebas substrate. Assay done in triplicate at each level. <sup>c</sup> Percentage of total amylase activity. <sup>d</sup> Calculated by use of the *t*-test for comparison of two means.

period, using the *t*-test for comparison of two means; they were not significantly different (*p* = 0.55, 0.81, and 0.81, respectively). Furthermore, the isoamylases pattern remained stable during this period.

**Sensitivity:** The two major isoenzymes P<sub>2</sub> and S<sub>2</sub> can be easily detected when total activity is as low as 5 U/L (sample 8, Figure 1). Since absent or low P-type isoamylases activity has been described in patients with chronic pancreatitis (14, 17, 18), such a degree of sensitivity may be useful.

**Linearity and activity of isoenzymes for each substrate:** Table 3 shows our results for isoenzyme quantitation on electrophoresing an equal mixture of P-type and S-type isoenzymes at three levels of total amylase activity. More of the P-type isoenzyme activity is accounted for than S-type activity. This is in good agreement with the findings of Stiefel and Keller (19), who showed that pancreatic amylase is more active on insoluble substrates than on soluble substrates. The reverse is true of parotid amylase. However, this difference gradually decreases with increasing total amylase activity, indicating a slight nonlinearity of the method in the working range of 0 to 100 U/L (sera with higher values are diluted). The result of these two opposite effects is a positive constant bias of about 4 U/L for P-type isoenzymes and a negative bias of the same magnitude for S-type isoenzymes when total amylase is between 25 and 100 U/L and when each fraction contributes 50% of the total activity. Because these biases are small, and abnormal results are at present best interpreted on a semi-quantitative basis (i.e., gross elevation of one type of isoenzymes or presence of an abnormal isoenzyme), they have no clinical significance. However, the difference in activity for various substrates should be kept in mind when one is comparing results obtained in different laboratories using the same technique for isoamylases and different techniques for total amylase.

### Isoamylases in Normal Individuals

Isoenzymes patterns found in normal individuals are illustrated in Figure 1. The P<sub>2</sub>S<sub>2</sub> pattern was found in 48 of 66 sera (73%), P<sub>1</sub>P<sub>2</sub>S<sub>2</sub> in 10 (15%), P<sub>2</sub>S<sub>2</sub>S<sub>3</sub> in seven (11%), and P<sub>2</sub> in one (1.5%). This is in harmony with the findings of others (11, 14, 20). The uncommon patterns already described (P<sub>1</sub>S<sub>2</sub>,

P<sub>1</sub>P<sub>2</sub>S<sub>2</sub>S<sub>3</sub>, and P<sub>1</sub>P<sub>2</sub>) were not found in this study, possibly because of a too-small population sample.

Reference values are presented in Table 4. No sex-related difference was found. On the average, about 40% of the activity in serum is ascribable to P-type isoenzymes and 60% S-type. Some authors have reported this proportion (5, 7, 21), but others found higher P-type activity (3, 14, 17). This can be due to difference in substrate, or to population differences, or both. Large inter-individual variation in the value of each isoenzyme was found. This is reflected by the wide range of reference values for both P-type and S-type isoamylases.

### Isoamylases in Pathological Conditions

Figure 2 illustrates patterns found in some pathological conditions. S-type isoenzymes showed high activity in mumps (sample 1). In acute pancreatitis, P-type isoenzymes were predominant and P<sub>3</sub> isoenzyme appeared (sample 2). P<sub>3</sub> isoenzyme was found only in serum from cases of acute pancreatitis or renal failure. These findings corroborate well those of Legaz and Kinney (22). Even after total amylase activity has returned to normal values, P<sub>3</sub> remains present for some time (sample 3). In macroamylasemia, a specific pattern can be found (sample 4) (11). Sample 5 is from a patient with pelvic inflammatory disease (acute salpingitis). Her total amylase activity was slightly above normal. The isoenzymogram shows the presence of S<sub>4</sub> isoenzyme, increase of S-type isoenzymes activity, and absence of an abnormal P-type isoenzyme.

The method presented is sensitive, precise, and allows separation of at least seven amylase isoenzymes. Patterns obtained in normal and abnormal conditions are comparable with those obtained by other investigators, especially those using electrophoretic separation on agarose (11, 13, 14) or on cellulose acetate (22). Furthermore, the method has the great advantage of being adaptable in most, if not all, clinical laboratories for routine use, because it requires only a simple apparatus and commercially available reagents, including agarose plates.

### References

1. Salt, W. B., and Schenker, S., Amylase—its clinical significance: A review of the literature. *Medicine (Paris)* 55, 269-289 (1976).
2. Warsaw, A. L., Serum amylase isoenzymes profiles as a differential index in disease. *J. Lab. Clin. Med.* 90, 1-3 (1977).
3. Otsuki, M., Saeki, S., Yuu, H., et al., Electrophoretic pattern of amylase isoenzymes in serum and urine of normal persons. *Clin. Chem.* 22, 439-444 (1976).
4. Berk, J. E., Shimamura, J., and Fridhandler, L., Tumor-associated hyperamylasemia. *Am. J. Gastroenterol.* 68, 572-577 (1977).
5. Berk, J. E., Fridhandler, L., and Montgomery, K. A., Isoamylase analysis: Methods and clinical application. In *Developments in Digestive Diseases*, Lea & Febiger, Philadelphia, PA, 1977, pp 133-152.
6. Berk, J. E., and Fridhandler, L., Clinical application of amylase isoenzyme analysis. *Am. J. Gastroenterol.* 63, 457-463 (1975).
7. Warsaw, A. L., and Lee, K. H., Characteristic alterations of serum isoenzymes of amylase in diseases of liver, pancreas, salivary gland, lung and genitalia. *J. Surg. Res.* 22, 362-369 (1977).
8. Lehrner, L. M., Ward, J. C., Karn, R. C., et al., An evaluation of the usefulness of amylase isoenzyme differentiation in patients with hyperamylasemia. *Am. J. Clin. Pathol.* 66, 576-587 (1976).
9. Levitt, M. D., Ellis, C. J., and Meier, P. B., Extraprostatic origin of chronic unexplained hyperamylasemia. *N. Engl. J. Med.* 302, 670-671 (1980).
10. Meites, S., and Rogols, S., Amylase isoenzymes. *CRC Crit. Rev. Clin. Lab. Sci.* 2, 103-138 (1971).
11. Skude, G., Electrophoretic separation, detection and variation of amylase isoenzymes. *Scand. J. Clin. Lab. Invest.* 13, 41-47 (1975).
12. Bermes, E. W., Eviti, V., and Forman, D. T., Statistics, normal values and quality control. In *Fundamentals of Clinical Chemistry*,

**Table 4. Reference Intervals Based on Values from 66 Healthy Individuals**

	Total amylase	P-type isoenzymes	S-type isoenzymes
	Activity, U/L	Activity, U/L	Activity, U/L
Mean	29.5	12.4	17.1
Reference interval	5-115	2-52	2-85

- N. W. Tietz, Ed., W.B. Saunders Co., Philadelphia, PA, 1976, pp 60-102.
13. Hobbs, J. R., and Aw, S. E., Urinary isoamylases. In *Enzymes in Urine and Kidney*, U. C. Dubach, Ed., Williams & Wilkins, Baltimore, MD, 1968, p 281.
  14. Magid, E., Horsing, M., and Rune, S. J., On the quantitation of isoamylases in serum and the diagnostic value of serum pancreatic type amylase in chronic pancreatitis. *Scand. J. Gastroenterol.* **12**, 621-627 (1977).
  15. Benjamin, D. R., and Kenny, M. A., Clinical value of amylase isoenzyme determination. *Am. J. Clin. Pathol.* **62**, 752-758 (1974).
  16. Gillard, B. K., Quantitative gel-electrophoretic determination of serum amylase isoenzymes distributions. *Clin. Chem.* **25**, 1919-1923 (1979).
  17. Skude, G., and Eriksson, S., Serum isoamylases in chronic pancreatitis. *Scand. J. Gastroenterol.* **11**, 525-527 (1976).
  18. Fahrenkrud, J., and Magid, E., Concentration of immunoreactive trypsin and activity of pancreatic isoamylase in serum compared in pancreatic diseases. *Clin. Chem.* **26**, 1573-1576 (1980).
  19. Stiefel, D. J., and Keller, P. J., Comparison of human pancreatic and parotid amylase activities on different substrates. *Clin. Chem.* **21**, 343-346 (1975).
  20. Laxova, R., and Kamaryt, J., Genetic aspects of human alpha amylase heterogeneity. In *Enzymes and Isoenzymes—Structure, Properties and Function (FEBS Symposium 18)*, D. Shugar, Ed., Academic Press, New York, NY, 1970, p 335.
  21. Levitt, M. D., Clinical use of amylase clearance and isoamylase measurements. *Mayo Clin. Proc.* **54**, 528-531 (1979).
  22. Legaz, M. E., and Kenny, M. A., Electrophoretic amylase fractionation as an aid in diagnosis of pancreatic disease. *Clin. Chem.* **22**, 57-62 (1976).