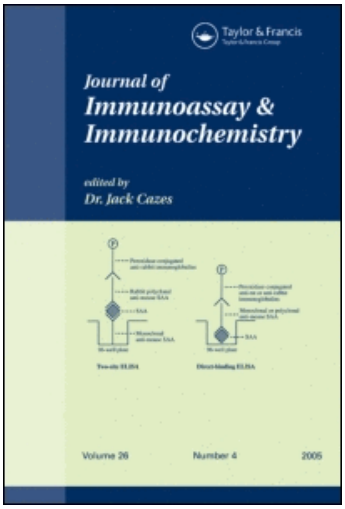

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Enzyme Immunoassay for Alpha-Amylase

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ENZYME IMMUNOASSAY FOR ALPHA-AMYLASE

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ABSTRACT

A sensitive sandwich enzyme immunoassay has been developed for quantitation of alpha-amylase in pig pancreas. The alpha-amylase-antibody conjugate was prepared from horseradish peroxidase coupled with antibodies to hog pancreas alpha-amylase. With this method the measuring range is 1-10 ng/ml and the detection limit is 0.2 ng/ml. The amount of pig pancreatic amylase was measured to be 6.21 $\pm$ 2.05 mg/g of tissue. The enzymatic activity determination and immunoassay were compared in the material studied.

KEY WORDS: alpha-amylase, pig pancreas, enzyme immunoassay.

INTRODUCTION

The determination of alpha-amylase activity in tissue homogenates and biological fluids is hindered by different factors which interfere with the assay. This fact, together with problems in substrate specificity, has prompted us to develop an immunological technique for measurement of amylase.

This paper describes a new sensitive enzyme-linked immunosorbent assay for quantitation of the amylase in pig pancreas.

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MATERIALS AND METHODS

Antiserum, Antibodies, Enzyme and Chemicals

Antiserum to pig pancreas alpha-amylase (Koch Light Laboratories, Colnbrook, Bucks, England) was obtained by the immunization of rabbits as reported previously (11).

The IgG was isolated from the antiserum or control serum by ammonium sulphate precipitation and DE-52 cellulose column chromatography, as described by Livingston (5). Specific antiamylase antibodies were obtained by affinity chromatography, using immobilized alpha-amylase as an immunosorbent (12). Purity of the preparations was verified by disc electrophoresis, immunodiffusion, and immunoelectrophoresis. Goat antirabbit IgG was obtained from Behringwerke AG, Marburg Lahn, West Germany. Freshly isolated pancreas from a healthy adult pig (Sus domestica) was homogenized with 4 vol. of 0.15 M NaCl in a Potter-Elvehjem glass homogenizer cooled with ice; the sediment was removed by centrifugation at 10.000 g for 10 min, and the supernatant used for the experiment. Horseradish peroxidase HRP (EC 1.11.1.7) was purchased from Serva Feinbiochemica, Heidelberg, West Germany.

Sephadex G-25 and Sepharose-Con A were the products of Pharmacia Fine Chemicals, Sweden. Polystyrene plates MRC were obtained from Linbro USA. Proteins were determined by the colorimetric method of Lowry et al (16), or, in the case of low protein concentrations, by the method of Blakesley and Boezi (13) with bovine serum albumin (Sigma Chemical Co., USA) used as the standard.

Alpha-amylase activity was determined by the method of Pick and Wober (9) using amylase from Koch Light Laboratories as the substrate. Horseradish peroxidase activity was assayed colorimetrically (14).

Preparation of Conjugate

Conjugate HRP-anti-alpha-amylase antibody was prepared using the two step method according to Avrameas and Ternynck (2). Briefly, 4.95 mg HRP dissolved in 100 ul phosphate buffer, pH 6.8, 100 mmol/L was reacted at 22°C for 18 h with glutaraldehyde (Serva) at a final concentration of 1.2%. A sample of modified HRP was separated from excess of glutaraldehyde by column filtration on Sephadex G-25. Fractions containing HRP were concentrated and allowed to react with 2.5 mg anti-alpha-amylase antibodies in carbonate buffer, pH 9.5, 50 mmol/L at 4°C for 24 h. Then 50 ul of 0.2 mol/L L-lysine was added, mixed and kept 2 h at 4°C. After dialysis against saline, the conjugate was precipitated with saturated ammonium sulphate. The conjugate was separated from free antibodies on a Con A-Sepharose column equilibrated in acetate buffer, pH 6.0, 100 mmol/L containing 1 mol/L NaCl, 1 mmol/L $MgCl_2$, and 1 mmol/L $CaCl_2$ (1). Fractions containing the conjugate were eluted by 100 mmol/L alpha-methyl D-glucoside (Sigma), concentrated to 0.2 ml, and kept at -12°C for over 6 months. In a double immunodiffusion test, antibody-peroxidase conjugate reacted with enzyme similarly to antibodies against alpha-amylase. Activity of the conjugate was unchanged during this period and reproducibility of the standard calibration curve was satisfactory.

ELISA Procedure

The procedure is summarized in Figure 1. Polystyrene plates were coated with 20 ug of antibody per bead in phosphate-buffered saline (PBS) at 25°C for 16 h. After incubation with gelatine 5 g/L at 37°C for 2 h, the plates were washed five times with gelatine 1 g/L in PBS. Standard solutions of alpha-amylase or samples containing enzyme were

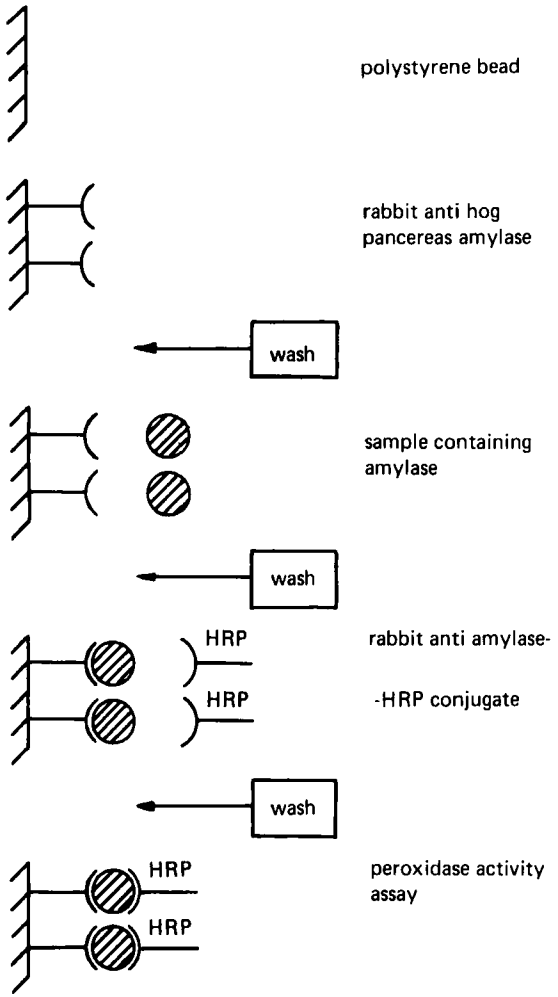

FIGURE 1: Sandwich ELISA procedure for amylase.

incubated with beads at 25°C for 2 h and overnight at 4°C. Plates were washed with gelatine 1 g/L and finally the beads were incubated with the conjugate solution, 400 ng per bead, at 4°C for 16 h. After washing, HRP activity was determined by the colorimetric method (14).

RESULTS

Characterization of Conjugate

Goat anti-rabbit IgG was used to separate free and covalently bound enzyme from conjugate. Determination of enzymatic activity showed 78% of peroxidase in the precipitate. With the quantity of enzyme covalently bound in the whole conjugate, and the quantity of enzyme before the conjugation, the yield was calculated to be 65%. The molar ratio of peroxidase to antibodies in the conjugate was determined to be 2.8. The loss of antibody reactivity due to conjugation was about 20% of the original.

Comparison of Amylase by Measurement of Enzymatic Activity and by ELISA

The amount of pig pancreas amylase was measured both by ELISA and by enzymatic assay using amylase as the substrate. A standard calibration curve of amylase is illustrated in Figure 2. For comparison, the enzymatic activity was converted to ug/L using the purified amylase for standard curve calibration. The mean amount of pancreatic amylase determined by ELISA in the 6 pigs was 6.21 ± 2.05 mg/g of tissue (mean ± S.D.). The content of amylase determined by enzymatic assay was 9.96 ± 3.88 mg/g of tissue (59.8 mg/g of homogenate protein). The amylase ratio determined by enzymatic assay and by ELISA was estimated to be 1.62.

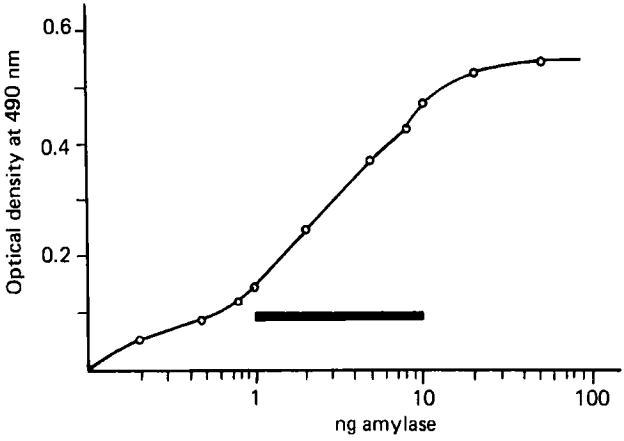

FIGURE 2: Standard calibration curve of amylase by the ELISA method. Horizontal bar-working range of the ELISA test.

DISCUSSION

Since the first communication about enzyme immunoassay was published by Engvall and Perlmann (4) in 1971, several assays for quantitation of serum proteins, antigens (including enzymes), hormones, and drugs have been published. During the last ten years many immunological studies of pancreatic and salivary amylases have been presented (7,8). Ryan et al (10) and Takatsuka et al (13) recently have developed specific radioimmunoassays for pig and human pancreatic amylases. Previous studies in our laboratory on the immunological properties of pancreatic amylase and purification of specific antibodies against amylase stimulated further investigations (11,12).

In the present paper, a specific and highly sensitive ELISA for pig pancreatic amylase is reported for the first time. The ratio of pancreatic amylase determined by colorimetric assay and ELISA was 1.62. These results may be explained by the effect of the complex kinetics of the catalyzed reaction and the lack of specific substrate for amylase.

Moreover, in the enzymatic assay, amylase content may be dependent on the presence of other pancreatic enzymes acting on amylase or its degradation products. It seems that use of the ELISA test has distinct advantages over the enzymatic assay. The ELISA method described here can be used even when alpha-amylase is inactivated or inhibited by low molecular compounds in biological material. The present results allow us to recommend this method for amylase assay.

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