ON THE MULTIPLE FORMS OF ACID PHOSPHATASE IN PIG LIVER

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1. Introduction

The existence of multiple forms of acid phosphatase (EC 3.1.3.2) in a single mammalian tissue is well documented. Several groups of workers have reported that gel filtration of tissue extracts yields two or three peaks of acid phosphatase activity, which appear to be distinct enzymes [1, 2]. The human prostatic enzyme, while giving a single peak on gel filtration [3, 4], exhibits heterogeneity when examined by electrophoresis [3, 4], isoelectric focussing [4], or ion-exchange chromatography [3, 4]. Smith and Whitby [3] observed a simplification of the electrophoretic band pattern of the human prostatic enzyme on treatment with neuraminidase, and Ostrowski and co-workers [4] have observed that after treatment with neuraminidase, the human prostatic enzyme gives a single peak on column isoelectric focussing. Direct analysis of highly purified samples of the human prostatic enzyme has demonstrated the presence of sialic acid, although some confusion appears to exist as to the actual amount present [4, 5].

There has been little work on the effect of neuraminidase on the multiple forms of other acid phosphatases, although Goldstone and co-workers [6] have shown that the electrophoretic mobilities of a number of rat kidney lysosomal hydrolases, including acid phosphatase, are reduced by treatment with neuraminidase. In the course of studies on the purification of acid phosphatase from pig liver, we investigated by several techniques the multiple forms of the enzyme that occur in this tissue, and have obtained a rather striking demonstration of the conversion of one form of the enzyme to another on neuraminidase treatment. In addition we have observed significant residual heterogeneity after neuraminidase treatment.

2. Methods

Pig liver chloroform/acetone powders were prepared as previously described [10]. Fresh pig liver was used immediately, or stored at -20° until required. The results obtained were not affected by freezing of the tissue. Aliquots of liver powder were extracted with 9 vol of ice-cold 0.10 M sodium acetate buffer, pH 4.90 at 25° (buffer A), for 30 min at 4°. After centrifugation, the clear supernatant was stored at 4° briefly until use. Fresh or frozen chopped liver was homogenized with 5 vol of ice-cold buffer A. The homogenate was then treated as for the powder extract.

Acid phosphatase activity was assayed by measuring the rate of release of *p*-nitrophenol from 1 mM *p*-nitrophenyl phosphate in buffer A containing 1 mM EDTA in a Cary 14 recording spectrophotometer with cell compartment thermostated at 25°.

Polyacrylamide gel isoelectric focussing was carried out in 6% cross-linked gels (0.5 X 15 cm) containing 1% Ampholine carrier ampholytes (pH range 3–10). The samples were set in the gels, which were subjected to a constant 310 V for 5 hr. The anode solution was 0.2% (v/v) H_2SO_4, the cathode solution 0.4% (v/v) ethanolamine. On extrusion, gels were stained for acid phosphatase activity by soaking at room temp. in buffer A containing 0.10 g of disodium α-naphthyl phosphate and 0.10 g of Fast Garnet GBC Salt per 100 ml [7]. When the bands were of the desired intensity, the gels were washed with several changes of buffer A, and photographed.

Neuraminidase (Worthington, *Clostridium perfringens*, 1.2 I.U. per mg) was dissolved in buffer A to a concentration of 1 mg/ml, and samples of acid phosphatase fractions were incubated with an equal

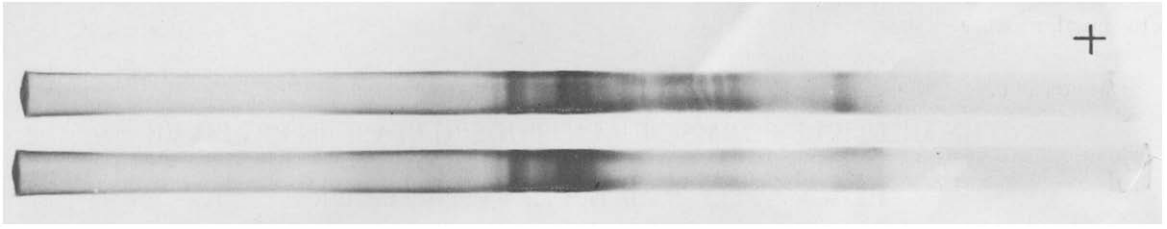

Fig. 1. Isoelectric focussing zymograms of pig liver and acid phosphatase fractions before and after treatment with neuraminidase; [Enzyme] = 0.4 I.U./ml. Top gel: 50 μl native enzyme; bottom gel: 50 μl native enzyme, after incubation with neuraminidase.

volume of this solution at 25° for 20 hr. In control experiments the neuraminidase was omitted.

Ammonium sulphate fractionation was carried out at 4° by adding 242 g/ℓ ammonium sulphate, with stirring, to the powder extract prepared as above. The extract was then stirred for 30 min and centrifuged at 3000 *g* for 30 min. The supernatant was decanted, adjusted to pH 8.0 with 1 M NaOH and a further 166 g/ℓ ammonium sulphate was added with stirring. After stirring for 30 min, the precipitate was collected by centrifugation at 3000 *g* for 30 min, redissolved in buffer A and dialyzed against several changes of buffer A containing 1 mM EDTA.

The dialysate from this step was further fractionated with acetone as follows: redistilled acetone at 0° was poured in a thin stream into the vigorously stirred enzyme solution until a concentration of 35% (v/v) was reached. The mixture was then stirred at 4° for 15 min, centrifuged at 0° for 30 min at 3000 *g* in stoppered cups, and the straw coloured supernatant decanted. Acetone at 0° was then added to the supernatant, raising the concentration to 55% (v/v), and the mixture was stirred and centrifuged as before. The supernatant was discarded and the precipitate resuspended in a small volume of buffer A containing 1 mM EDTA, dialyzed against several changes of the same buffer at 4°, centrifuged free of an appreciable precipitate, and stored at 4°.

CM-Sephadex C-50 was swollen in distilled water, treated successively with 1 M NaCl, 0.05 M NaOH, and column buffer (buffer A containing 1 mM EDTA). A column (5 × 90 cm) of the resin was poured, and equilibrated at 4° with column buffer. Linear concentration gradients of NaCl in column buffer were applied in the usual way.

A column (4.5 × 87 cm) of Sephadex G-100 was packed, and elution was carried out with buffer A containing 1 mM EDTA.

3. Results

Fig. 1 shows the results of gel isoelectric focussing of an extract of a pig liver chloroform/acetone powder

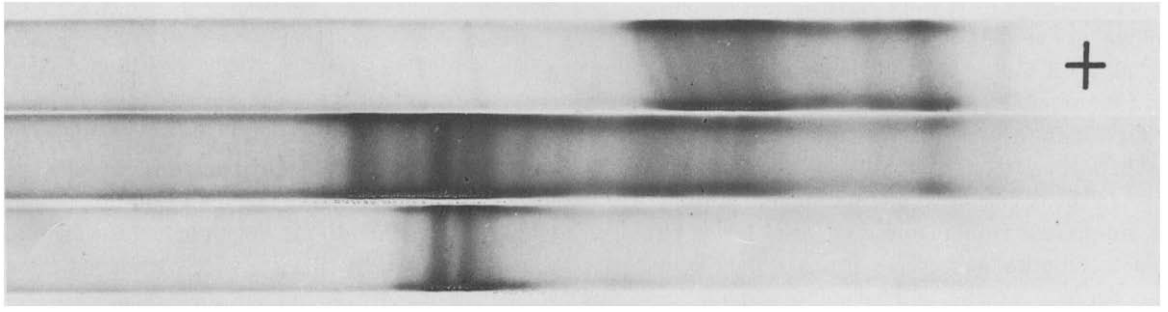

Fig. 2. Isoelectric focussing zymograms of pig liver acid phosphatase fractions before and after neuraminidase treatment. Top gel: 50 μl acid phosphatase fraction not bound to CM-Sephadex; [Enzyme] = 1.48 I.U./ml. Middle gel: 50 μl native enzyme; [Enzyme] = 0.4 I.U./ml. Bottom gel: as for top gel, after incubation with neuraminidase.

before and after neuraminidase treatment. The most anodic enzyme bands were markedly reduced in intensity by neuraminidase. Fresh or frozen liver extracts gave identical results.

Pig liver chloroform/acetone powder (1200 *g*) was extracted, fractionated with ammonium sulphate and acetone and applied to the column of CM-Sephadex. The column was then washed with buffer A containing 1 mM EDTA. A considerable amount of protein, and a small amount of acid phosphatase activity were not bound. Some of this material was concentrated, and gel isoelectric focussing of this material showed that it consisted of the most anodic bands present in crude extracts (fig. 2). On treatment with neuraminidase, this material reverted to a pattern identical with the more anodic of the two groups of bands that remained after treatment of crude extracts with neuraminidase (fig. 2).

After extensive washing of the CM-Sephadex column, the bound material was eluted with a linear salt gradient (0–0.6 M NaCl in column buffer); two further discrete peaks of activity were eluted. On gel isoelectric focussing, the first peak corresponded to the more anodic of the two groups of bands remaining after neuraminidase treatment of crude extracts, the second peak to the more cathodic group. Neuraminidase treatment of these two enzyme fractions did not affect their band patterns. Gels containing neuraminidase alone gave no acid phosphatase bands.

When the gel staining mixture contained 10 mM (+)-tartrate, no bands appeared in normal staining times in gels containing fresh liver or powder extract. On gel filtration of 40 ml of powder extract on the Sephadex G-100 column, two widely separated peaks of acid phosphatase activity were observed. When the material in the peak of low molecular weight was concentrated, subjected to gel isoelectric focussing, and staining for acid phosphatase activity was attempted, no bands could be detected. Previous workers have shown that α-naphthyl phosphate is a poor substrate for the low molecular weight acid phosphatases from human placenta [8], and horse erythrocytes [9].

The activities of all three CM-Sephadex fractions towards *p*-nitrophenyl phosphate were strongly inhibited by the presence of 1 mM (+)-tartrate or 1 mM fluoride in the assay mixture; the low molecular weight enzyme from Sephadex G-100 chromatography of powder extract was, in contrast, unaffected by 1 mM (+)-tartrate, and only slightly inhibited by 1 mM fluoride.

The activity against *p*-nitrophenyl phosphate of the material applied to CM-Sephadex above was almost completely inhibited by (+)-tartrate, in contrast to the original extract, the activity of which was only inhibited ca. 60%. Clearly, the low molecular weight enzyme is lost during the ammonium sulphate and acetone fractionations of our purification procedure. All the bands observed in isoelectric focussing gels occur in the peak of high molecular weight observed on Sephadex G-100 chromatography.

4. Discussion

Pig liver lysosomal acid phosphatase gives rise to a very complex band pattern on analytical gel isoelectric focussing. On treatment with neuraminidase the most acidic bands are lost. Neuraminidase treatment of the most acidic phosphatase fractions from CM-Sephadex chromatography showed that these multiple anodic forms of the enzyme are in fact converted to forms apparently identical with one of the two activities obtained from CM-Sephadex gradient chromatography. These two remaining groups were separable by CM-Sephadex chromatography, each giving a single peak, and their gel isoelectric focussing patterns were unaffected by neuraminidase. In contrast to this, isoelectric focussing of the human prostatic acid phosphatase after neuraminidase treatment reportedly gives a single peak of activity, although the enzyme is highly heterogeneous prior to neuraminidase treatment [4]. This work therefore demonstrates that while sialic acid attachment contributes markedly to the observed heterogeneity, other factors may also be involved.

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