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Skeletal-muscle α-glucosidases in bovine generalized glycogenosis type II

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The skeletal muscle of cattle suffering from generalized glycogenosis type II was shown to lack acid α-glucosidase (EC 3.2.1.3) activity. Furthermore, there was no evidence of enzymically inactive proteins that cross-reacted with antibodies raised against acid α-glucosidase from the muscle of normal animals.

Generalized glycogenosis type II is an inherited, lethal, lysosomal storage disease of humans (Baudhuin *et al.*, 1964), which has also been described in Shorthorn cattle (Richards *et al.*, 1977). The disease has been shown in humans (Hers, 1963, 1965) and Shorthorn cattle (Jolly *et al.*, 1977; Howell *et al.*, 1981) to be due to decreased activity of lysosomal α-glucosidase (acid maltase, EC 3.2.1.3) that results in widespread accumulation of glycogen in tissues.

Desnick *et al.* (1976) proposed a number of heritable mutations that could result in the enzymic defects seen in many inherited diseases. They also related these mutations to the presence or absence of material, in tissues from affected individuals, that would cross-react with antibodies raised against the respective enzymes from normal animals (cross-reacting material).

Tissue homogenates of calves suffering from generalized glycogenosis type II retain a small α-glucosidase activity (about 3% of normal) when assayed at pH 4.0 (Richards *et al.*, 1977; Howell *et al.*, 1981). The character of this 'residual' activity and the type of mutation present in bovine glycogenosis type II were the subject of the present investigation.

Materials and methods

α-Glucosidase activity was determined fluorimetrically with 4-methylumbelliferyl α-D-glucopyranoside (Koch–Light) as substrate. Each reaction mixture consisted of 100 μl of a suitably dilute enzyme sample, plus 100 μl of substrate in 0.2 M-citrate/phosphate buffer, pH 4.0 or 6.5, to give a final substrate concentration of 2.5 mM. The mixtures were incubated for 30 min at 37°C and the reaction was terminated by the addition of 2.0 ml of 0.5 M-glycine/NaOH buffer, pH 10.4. Fluorescence of liberated 4-methylumbelliferone was measured on a Farrand Mk. 1 spectrofluorimeter with an excitation wavelength of 350 nm and an emission wavelength of 440 nm. One unit of activity was defined as 1 μmol of product/min. The above reaction mixture was appropriately modified to determine the effect of pH and substrate concentration on α-glucosidase activity.

Muscle was taken from affected animals which were in the terminal stages of the disease, at approx. 12–15 months of age. A rear leg was amputated under deep pentobarbitone anaesthesia and the remainder of the animal was perfused with glutaraldehyde to fix the tissues for microscopy. Age-matched, clinically normal, animals were treated similarly. Muscle was cut into 200–300 g portions, frozen and stored at −20°C until required.

Approx. 300 g of frozen muscle was homogenized (20%, w/v) in 10 mM-potassium phosphate buffer, pH 6.8, containing 0.01% NaN_3 (standard buffer) in a 5-litre stainless-steel Waring blender. The homogenate was centrifuged at 26000 $g_{max.}$ for 30 min in 300 ml polycarbonate bottles in a GSA rotor of a Sorvall RC5 refrigerated centrifuge. The supernatant (approx. 1000 ml) was chromatographed at 4°C on a column (15 cm × 0.9 cm diam.) of concanavalin A–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) which had been equilibrated with standard buffer. The sample was applied at 60 ml/h, the column was washed with 50 ml of standard buffer and the absorbed material was eluted with 500 ml of 0.5 M-methyl α-D-mannopyranoside in standard buffer (30 ml/h). Collected fractions (10 ml) were assayed for α-glucosidase activity at pH 4.0 and 6.5. The eluted active fractions were pooled, concentrated and washed with standard buffer over an Amicon PM-10 filter.

Concentrated, washed samples (20 ml) from concanavalin A–Sepharose chromatography (approx. 50 mg of protein) were applied to a Sephadex G-100 (Pharmacia) column (60 cm × 2.6 cm diam.) equilibrated with standard buffer. Samples were chrom-

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atographed with 1 litre of standard buffer at 12ml/h, and 10ml fractions were collected. Fractions were assayed for α-glucosidase activity at pH4.0 and 6.5. The resultant chromatograms were essentially the same as those reported by other workers (Bruni *et al.*, 1969; Palmer, 1971; Murray *et al.*, 1978), where acid α-glucosidase was retarded by the Sephadex column, but not bound (requiring maltose-facilitated elution), like human placental acid α-glucosidase reported by de Barsy *et al.* (1972) or that from cultured human fibroblasts reported by Beratis *et al.* (1978). Fractions required for further study were pooled and concentrated.

Pooled, concentrated, fractions from Sephadex G-100 (approx. 5mg of protein) or concanavalin A–Sepharose chromatography (approx. 50mg of protein) were further chromatographed on DEAE-Sepharose (Pharmacia). Samples (20ml) were applied to a column (70cm × 1.6cm diam.) equilibrated with standard buffer at 4°C. The sample was eluted with a 1 litre linear NaCl gradient (0–1 M) in standard buffer. The flow rate was 60ml/h, and collected fractions (10ml) were assayed for α-glucosidase activity at pH4.0 and 6.5.

Acid α-glucosidase prepared from normal bovine skeletal muscle by concanavalin A–Sepharose, Sephadex G-100 and DEAE-Sepharose chromatography was used as the antigen for inoculation of guinea pigs. Two acid α-glucosidase preparations (specific activities 2.2 and 2.5units/mg of protein) were pooled and washed with distilled water over an Amicon PM-10 membrane filter, then freeze-dried and stored for later use. The preparation was found to be homogeneous by polyacrylamide-gel electrophoresis on Pharmacia PAA 4/30 gradient gels run in 0.09M-Tris/0.08M-borate/0.003M-EDTA buffer, pH8.4, for 15h at 125V. Enzyme protein (840μg) was dissolved in 2ml of 0.9% NaCl and emulsified with an equal volume of complete Freund's adjuvant (Commonwealth Serum Laboratories, Melbourne, Vic., Australia). Six guinea pigs received 50μl injections in each foot pad, followed 2 weeks later by 50μl injections in the rear foot pads and after another 10 days by a final intramuscular injection of 200μl, All guinea pigs were bled by cardiac puncture 10 days after the last injection. The blood was allowed to clot at 4°C overnight, and the serum was collected after centrifugation at 2000*g* for 10min. Four of the six guinea pigs produced antibodies to bovine acid α-glucosidase.

Immunoprecipitation experiments were performed by the following procedure. Suitably diluted purified acid α-glucosidase from muscle of normal cattle (100μl) was mixed with 0–100μl of diluted antiserum in a 400μl Beckman Microfuge tube and made up to 200μl with 10mM-phosphate buffer, pH6.8, containing 0.5M-NaCl. Standard buffer (100μl) or 100μl of solutions to be tested for cross-reacting material was added. After incubation overnight at 4°C, tubes were centrifuged in a Beckman Microfuge B for 5min and 100μl of the supernatant was assayed for α-glucosidase activity at pH4.0 and 6.5.

Immobilized antibodies were prepared by linkage to Sepharose 4B. γ-Globulins precipitable from serum by 50% saturation with $(NH_4)_2SO_4$ were redissolved in 0.1M-$NaHCO_3$, pH8.3, containing 0.5M-NaCl to give a protein concentration of approx. 10mg/ml. This solution was added to reconstituted CNBr-activated Sepharose 4B (Pharmacia) in the ratio 2:1 (v/v) and agitated for 2h at room temperature (28°C). Remaining active groups were then blocked by transferring the gel to 2vol. of 0.2M-glycine buffer, pH8.0, and agitating for 2h at room temperature. The gel was then washed on a sintered-glass filter with alternately 0.1M-$NaHCO_3$ buffer (pH8.3)/0.5M-NaCl and 0.1M-acetate buffer (pH4.0)/0.5M-NaCl. This cycle was repeated five times. The gel was finally washed and stored in 10mM-phosphate buffer, pH6.8, containing 0.5M-NaCl and 0.1% NaN_3.

Results and discussion

Tissues of calves suffering from generalized glycogenosis type II contain a low α-glucosidase activity at pH4.0 (Richards *et al.*, 1977; Howell *et al.*, 1981). In isolation, this suggests that the nature of the genetic defect lies in the production of a defective enzyme with low specific activity or decreased stability or in a disturbance of the mechanism that regulates the activity of a structurally normal enzyme. Our results show that none of these possibilities is correct.

Chromatography of bovine muscle α-glucosidase on concanavalin A–Sepharose showed that the glycoprotein acid α-glucosidase bound to the column, whereas a neutral α-glucosidase passed through (Fig. 1*a*). This is similar to the behaviour of the neutral and acid α-mannosidases on concanavalin A–Sepharose (Phillips *et al.*, 1976). Thus we used concanavalin A–Sepharose to isolate and concentrate lysosomal acid α-glucosidase from bovine muscle and to attempt to concentrate the low 'residual' α-glucosidase activity in affected muscle when assayed at pH4.0.

Extracts of muscle from normal or affected animals both contained α-glucosidase activity that bound to concanavalin A–Sepharose and was eluted by methyl mannoside (Fig. 1). There was, however, a marked difference in the pH optimum of the enzymes eluted in each case. In the preparation from normal muscle, most activity was present when assayed at pH4.0, with some detectable at pH6.5 (Fig. 1*a*). On the other hand, in that from affected muscle there was no activity at pH4.0, but there was

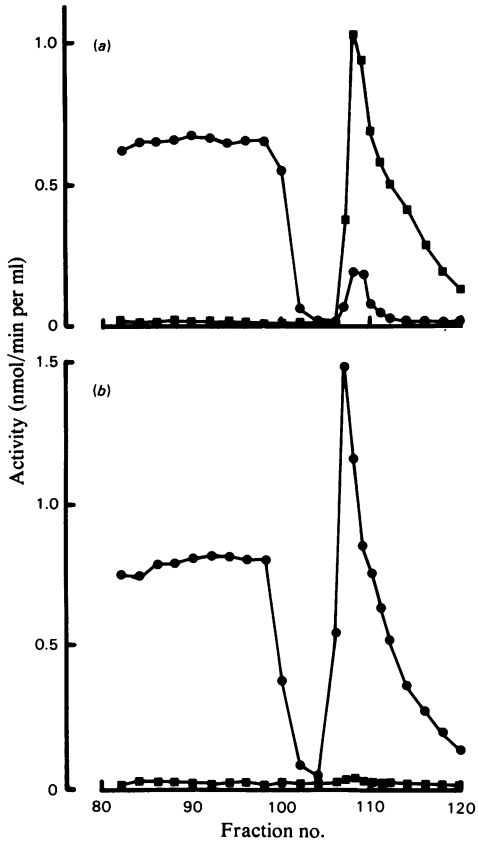

Fig. 1. *Chromatography of bovine skeletal-muscle α-glucosidase on concanavalin A–Sepharose*

The supernatants of 20% (w/v) homogenates in 10mM-phosphate buffer, pH6.8, containing NaN_3, of (*a*) normal muscle or (*b*) affected muscle were applied to the column. Approx. 1000ml of supernatant was applied in each case and 10ml fractions of the last 160ml that passed through the column were collected for assay. The column was then washed with buffer (fractions 98–104) and eluted with the same buffer containing 0.5M-methyl α-D-mannopyranoside. Collected fractions were assayed for α-glucosidase activity at pH6.5 (●) and pH4.0 (■).

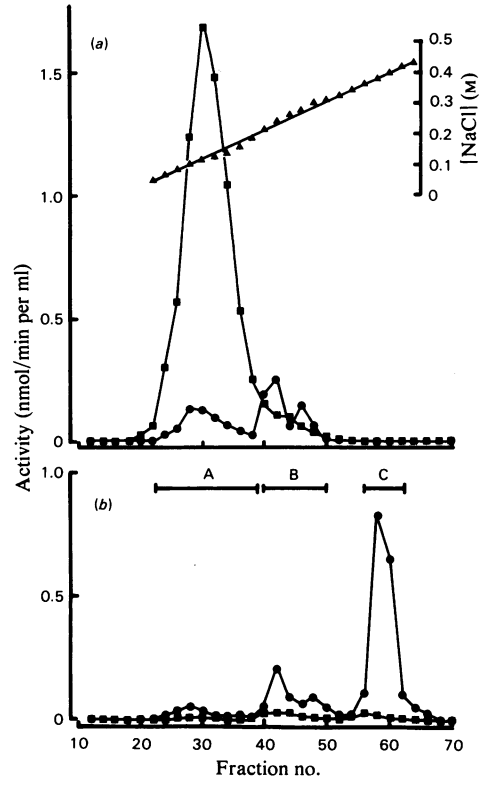

Fig. 2. *Chromatography of bovine skeletal-muscle α-glucosidase on DEAE-Sepharose*

Material eluted from concanavalin A–Sepharose after chromatography of α-glucosidase activity from (*a*) normal muscle or (*b*) affected muscle was applied to the column and eluted with a NaCl gradient (▲). Collected fractions (10ml) were assayed for α-glucosidase activity at pH6.5 (●) and pH4.0 (■). Peaks A, B and C are discussed in the text.

high activity at pH6.5 (Fig. 1*b*). In both cases a neutral α-glucosidase passed through the column.

When the respective methylmannose eluates were chromatographed on DEAE-Sepharose, the absence of activity at pH4.0 in the preparation from affected muscle was confirmed (Fig. 2*b*). In addition, it was revealed that most of the activity at pH6.5 in such preparations chromatographed in a different position (peak C) from that in the preparation from normal muscle (Fig. 2*a*). The material in peak B had a broad pH profile and at pH4.0 retained 15–20% of its activity at the optimal pH, 6.5, whereas the enzyme in peak C had no detectable activity at pH4.0. Material in peak A from normal muscle had a pH optimum between 4.0 and 4.5 and retained approx. 20% of its maximal activity when assayed at pH6.5. Acid α-glucosidase activity, which was retarded on Sephadex G-100, co-chromatographed with peak A on DEAE-Sepharose.

The complete lack of acid α-glucosidase activity from muscle of affected animals has led to the conclusion that the apparent 'residual' acid α-glucosidase activity in tissue homogenates is due to the activity at pH4.0 of neutral α-glucosidases that have a broad pH profile. This is in contrast with the findings of Burditt *et al.* (1978, 1980), who have shown that in human, bovine and feline mannosidosis a defective acid α-mannosidase with an

increased K_m and decreased stability is synthesized. The residual activity seen in these cases can be explained by the presence of this defective enzyme, which like normal acid α-mannosidase can be concentrated on concanavalin A–Sepharose.

Antiserum against acid α-glucosidase was used to determine whether cross-reacting material was present in affected muscle. A 1 ml sample of pooled antisera, prepared for this study, precipitated 0.05 unit of acid α-glucosidase activity. It also precipitated acid α-glucosidase from bovine liver, kidney, brain and heart, but did not affect any of the neutral α-glucosidase activities from these tissues. In immunoprecipitation experiments, the addition of material prepared from affected muscle did not influence the capacity of the antibodies to precipitate acid α-glucosidase activity, thereby suggesting that affected muscle does not contain cross-reacting material. The preparations tested included 20% (w/v) muscle supernatant, material passed through and eluted from concanavalin A–Sepharose and the various fractions from the DEAE-Sepharose chromatography. A 1 ml column of immobilized antibodies bound acid α-glucosidase activity from extracts of normal muscle, but allowed all neutral α-glucosidase activity to pass through. The same column, fully loaded with acid α-glucosidase and thoroughly washed with standard buffer, did not release any α-glucosidase activity when eluted with extracts from affected muscle, although activity was readily released by 0.04 M-citrate buffer, pH 3.0, once again indicating a lack of cross-reacting material. This finding is consistent with those of de Barsy *et al.* (1972), Dreyfus *et al.* (1974) and Murray *et al.* (1978), who were not able to detect cross-reacting material in tissues of human patients suffering from glycogenosis type II. However, this is in conflict with the work of Beratis *et al.* (1978), who reported the presence of cross-reacting material in cultured fibroblasts of human patients suffering from the infantile and the adult forms of glycogenosis type II.

It is tempting to conclude that the absence of immunoreactive material excludes the possibility that a mutated inactive enzyme may have been present in the tissues of our affected cattle. However, de Barsy *et al.* (1972) showed that alkaline inactivation of human acid α-glucosidase also destroyed the ability of the enzyme to react with its antibodies. This suggests that even a small conformational change may be expected to affect the antigenicity of α-glucosidase and that a mutant enzyme might also lose its cross-reacting ability.

The significance of the neutral α-glucosidase (peak C) in affected muscle is not fully understood by us, but by the methods employed here (concanavalin A–Sepharose and DEAE-Sepharose chromatography) it is present at relatively high activity in the brain, kidney and liver of normal cattle and is also found in muscle of newborn calves. We do not consider glycogenosis itself to be responsible for the presence of this activity in affected muscle, but it may reflect a secondary change, e.g. increased myoblastic activity.

In conclusion, we therefore suggest that in generalized glycogenosis type II of Shorthorn cattle, the gene coding for acid α-glucosidase either is not transcribed or contains a mutation which results in the complete loss of enzymic and cross-reacting activity of the mutant gene product.

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