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Ox-spleen β-Glucuronidase; its Purification and a Study of some Factors Involved in Assaying its Activity

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Masamune (1934) showed that aqueous extracts of ox kidney contained an enzyme which appeared to be specific in catalyzing the hydrolysis of β-glucuronides at the glucosidic link. The method of purification developed by Masamune for this 'β-glucuronidase' involved autolysis of the kidney with water and precipitation of the enzyme with ethanol. Oshima (1936) carried the purification a stage further than Masamune by adsorbing the enzyme on kaolin and subsequently eluting it with sodium phosphate. In repeating the work of the Japanese investigators Fishman (1939*a*, *b*) found difficulty in obtaining active enzyme preparations and devised an entirely new method for its purification from ox spleen. In this method acetone precipitation of the enzyme from the aqueous spleen extract was followed by an isoelectric precipitation of inactive protein. The enzyme preparation was further purified by fractionation with various concentrations of ammonium sulphate.

In view of the suggestion by Fishman (1940) that β-glucuronidase might catalyze the synthesis of conjugated glucuronides in the animal body, its chemical behaviour merits further study. The present research was therefore initiated with the ultimate intention of carrying out a systematic physicochemical examination of β-glucuronidase action. During the preliminary work Fishman's method (1939*a*) of purifying the enzyme from ox spleen was repeated. Since it was found that a large loss in activity occurred as a result of the acetone precipitation a different method was devised and is described below. Some factors involved in the estimation of β-glucuronidase activity in spleen extracts were investigated and are also described.

EXPERIMENTAL

The estimation of β-glucuronidase activity

Bio-synthetic *l*-menthol glucuronide prepared by the method of Williams (1938) was crystallized three times from water. The substrate solution was prepared by suspending 500 mg. *l*-menthol glucuronide ($C_{16}H_{28}O_7.1{\cdot}5H_2O$) in about 10 ml. water. A few drops of N-NaOH were added and the mixture was warmed to effect solution. After cooling it was titrated electrometrically to pH 5·0 with N-NaOH and diluted to 25 ml.

Enzyme (0·50 ml.) was added to acetate buffer (0·10M, 1·0 ml., pH 5·0) and substrate (0·50 ml.) in a 15 ml. pyrex centrifuge tube which was stoppered with a rubber bung and incubated for 2 hr. at 25°. The protein was then precipitated, centrifuged down, and the reducing power of a suitable measured sample of supernatant liquid was determined by Levvy's modification (1946) of the cerimetric method. A control tube in which enzyme solution, buffer and water were placed in the same proportion as above was treated in an identical manner. Since the ceric sulphate was standardized against glucurone the amount of reducing material found in each tube was calculated in terms of glucuronic acid by applying a correction factor. From the difference between the two estimations the amount of glucuronic acid liberated by 1 ml. of enzyme solution was calculated.

Under the conditions of the activity measurement it was found that the time-action curve was of zero order. If the concentration of the enzyme solution used for assay was adjusted so that the amount of substrate hydrolyzed was less than 10 %, the rate of hydrolysis was directly proportional to the enzyme concentration. It is therefore proposed to define one β-glucuronidase unit (G.U.) as that amount of enzyme which will liberate 0·100 mg. of glucuronic acid from the substrate *l*-menthol glucuronide under the conditions specified.

Liberation of reducing substance from ox-spleen extract

During the preliminary work on the purification of the enzyme, Fishman's practice (1939*a*) of controlling the enzyme assay by adding substrate to boiled enzyme was followed. However, under the conditions for estimating enzyme action in the present investigation, the amount of substrate hydrolyzed was rather small and results obtained for β-glucuronidase activity of a spleen extract on several successive days were often erratic. It soon appeared that this type of control was unsuitable since dialyzed aqueous extracts of acetone-treated spleen (described in detail later, stage A, Table 1), when incubated alone formed a relatively large amount of reducing substance which was estimated in the ceric sulphate titration.

Enzyme solution previously heated to 100° for 3 min. did not liberate any reducing material on incubation at 25°. At any time during incubation of the enzyme extract at 25°, the reducing material which had been formed was not destroyed by heating for 3 min. at 100°. In most cases the amount of reducing material formed during incubation of the enzyme at 25° was many times greater than the amount of glucuronic acid formed when substrate was present. Consequently little reliance could be placed on the figure obtained for β-glucuronidase activity of the spleen extracts. One stage of purification did, however, remove a large proportion of the precursor of this reducing substance; the error consequently was most serious in the assay of the initial aqueous extract.

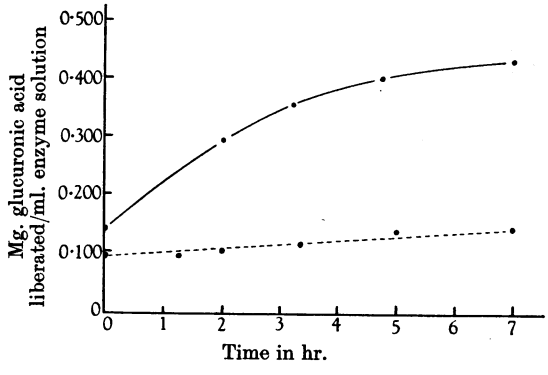

Fig. 1. Liberation of reducing material from aqueous extract of acetone-treated ox spleen by incubation at 25°. Solid line: spleen extract; broken line: after dialysis of extract incubated at 25° for 7 hr.

The solid line in Fig. 1 shows the time-action curve for liberation of reducing material by a spleen extract (A, Table 1) over a 7 hr. period at 25° and pH 5·0. The rate of release of reducing material during reincubation, after 24 hr. dialysis, is represented by the broken line. (The reducing substance was treated as if it were glucuronic acid and is plotted as such in Fig. 1.)

It is seen that the reducing material formed during the incubation period was removed by dialysis and that there was little further liberation of this material on subsequent incubation at 25°. Similar results were obtained with several different spleen extracts. In some cases the production of reducing material was virtually complete after 5 hr. incubation, in others as much as 8 hr. were required for completion of the process. A method was thus furnished for avoiding the high blank that would otherwise be obtained in assaying the activity of the initial spleen extract. When spleen was not subjected to the preliminary acetone treatment, the amount of reducing material formed on incubation of an aqueous extract was much greater than in extracts prepared after acetone treatment of the tissue.

Purification of β-glucuronidase

Throughout the procedure pH was controlled with a glass electrode. At each step in the purification enzyme activity was determined. Protein nitrogen (P.N.) was precipitated from samples of enzyme solution by addition of an equal volume of 10% trichloroacetic acid (w/v) and estimated by the micro method of Ma & Zuazaga (1942). The ratio G.U./mg. P.N. for an enzyme solution has been used as a basis for calculating purity after each stage of the purification. Figures for activity and P.N. at each stage in the purification are given in Table 1 for a representative preparation.

Table 1. *Purification of β-glucuronidase; activity, protein nitrogen and purity at various stages of a representative preparation*

Enzyme solution: Stage of purification	Volume (ml.)	Activity/ml. (G.U.)*	Total activity (G.U.)*	Protein-N (mg./ml.)	Activity/mg. protein-N (G.U.)*
A	2,365	0·61	1,432	2·16	0·28
B	2,365	1·27	3,050	1·81	0·70
C	2,440	1·21	2,960	0·58	2·10
D	243	10·5	2,550	3·45	3·04
E	63	40·8	2,570	3·52	11·62
F	19	100	1,900	3·58	30·0
G	21	92·5	1,765	2·68	34·5
Combined with other preparations at a similar stage of purification					
H	77	174	13,400	3·28	53·2
K	39	247	9,640	2·80	88·4

* See p. 603.

Stage A. Two fresh ox spleens were stripped of fat and minced (1700 g.). Anhydrous redistilled acetone (3400 ml.)

was stirred into the mince and the mixture was filtered with suction through a soft paper. After repeating the acetone treatment on the filter cake, the latter was broken up and stirred mechanically for 1 hr. with tap water (3400 ml.). After centrifugation the supernatant liquid containing the enzyme was collected and dialyzed overnight against running tap water (A).

Although the precipitation of β-glucuronidase from aqueous spleen extract with acetone led to a loss in activity it was found that minced spleen washed with acetone and then extracted with water yielded about the same quantity of enzyme as the untreated spleen. Such preliminary acetone treatment was found to be advantageous, since it reduced the total protein in the aqueous extract to about one-third.

Stage B. The dialysate (A) was adjusted to pH 5·0 with 4N-acetic acid, when a heavy precipitate formed; 4M-acetate buffer (5 ml./l., pH 5·0) was added and after addition of a few drops of toluene the solution was incubated at 25–30° for 6 hr. (B).

During the incubation period a large amount of dialyzable reducing material was liberated which interfered with the measurement of enzyme activity. Activity and P.N. estimations were carried out on a measured sample of B which had been dialyzed overnight. As is seen from Table 1 removal of the reducing material by dialysis led to a large increase of β-glucuronidase activity; this phenomenon will be discussed later. There was no need to dialyze the main bulk of solution free of reducing material, since later precipitation of the enzyme with ammonium sulphate removed it from the preparation.

Stage C. In order to remove the precipitate of inactive protein which had formed at pH 5·0, 'Standard Super Cel' (25 g./l.), then 'Celite 545' (35 g./l.) (Johns-Manville Corp. filter aids) were stirred into solution B and the mixture was filtered with light suction through a soft paper. The cake was washed with water (200 ml.) and discarded. The combined filtrate and washings, containing the enzyme, was a clear deep red solution (C). A measured sample was dialyzed before carrying out activity and P.N. estimations.

Stage D. Solid ammonium sulphate (215 g./l.) was stirred into solution C. After standing 10 min., 'Standard Super Cel' (10 g./l.), then 'Hyflo Super Cel' (15 g./l.) were stirred in, the mixture was filtered with suction and the cake discarded. The filtrate was made up to 450 g./l. with solid ammonium sulphate and allowed to stand overnight in an ice-chest. Most of the clear supernatant liquid was then siphoned off and the remainder centrifuged. The cake, containing the enzyme, was dissolved in the minimum quantity of water and dialyzed free of ammonium sulphate in running tap water (D).

Little purification was achieved by this step, but a concentrated protein solution was obtained which enabled the later ammonium sulphate fractionation to be carried out efficiently. Removal of inactive protein precipitates has been found to be greatly facilitated by addition of 'Celite' filter aids. Active precipitates, however, were better separated by centrifugation; although they could be filtered off with 'Celite', and could be eluted quantitatively with water, the resulting solutions were more dilute than was desirable for further purification.

In the remaining stages of the procedure advantage was taken of the finding that the variation in solubility of the enzyme at pH's between 4·3 and 7·3 was different from that of much of the inactive protein. Ammonium sulphate fractionation was therefore carried out at three different pH's between these limits.

Stage E. Solution D was adjusted to pH 5·0 with 4N-acetic acid and sufficient SAS (saturated ammonium sulphate solution), previously brought to pH 5·0 with 3N-NaOH, was added to bring the salt concentration to 0·35 SAS. 'Standard Super Cel' (10 g.) was stirred in, the precipitate filtered off at the pump, washed with a few ml. 0·35 SAS and discarded. SAS was added to the combined filtrate and washings at pH 5·0 until it was 0·50 saturated, the resulting enzyme precipitate being centrifuged down, dissolved in water and dialyzed (E).

Stage F. Solution E was made 0·20 saturated with SAS and titrated to pH 4·3 with 4N-acetic acid. A small precipitate was filtered off with suction after addition of 'Standard Super Cel' (1 g.) and discarded. The filtrate was titrated to pH 5·0 with 3N-NaOH and made 0·35 saturated with SAS. The precipitate was filtered off with suction after addition of 'Standard Super Cel' (1 g.), washed with a small amount of 0·35 SAS and discarded. 3N-NaOH was added to the filtrate until the pH was 7·3, when a heavy precipitate formed. The mixture was made 0·36 saturated with SAS, previously titrated to pH 7·3, 'Standard Super Cel' (1 g.) added, and the mixture filtered with suction. The cake was washed with a few ml. 0·36 SAS (pH 7·3) and discarded. SAS was added to the filtrate at pH 7·3 until it was 0·46 SAS, the precipitated enzyme was centrifuged off, dissolved in water and dialyzed free of ammonium sulphate against distilled water (F).

Stage G. After 24 hr. dialysis a flocculent precipitate was centrifuged off and discarded (supernatant solution G).

Stage K. Solution G was combined with several other preparations worked up to the same stage to give solution H. This was adjusted to pH 5·0 with 4N-acetic acid made 0·35 saturated with SAS; a heavy precipitate was filtered off with 'Standard Super Cel' (2 g.) and discarded. The filtrate was made 0·36 SAS at pH 7·3, 'Standard Super Cel' (1 g.) was added and the mixture filtered. SAS was added to the filtrate at pH 7·3 until it was 0·46 saturated, the precipitate containing the enzyme being centrifuged off, dissolved in water and dialyzed against distilled water to give a clear deep brown solution (K).

The value of G.U./mg. P.N. for solution K shown in Table 1 is one of the highest that has yet been obtained. It is seen that the activity/mg. P.N. of this solution is about 125 times greater than that at stage B. If the increase in activity resulting from incubation of the original aqueous spleen extract is taken into consideration the activity/mg. P.N. at stage K is about 315 times that at stage A. Many purified preparations have been made by this method and in general the purification resulting from each step follows closely that shown for the single preparation represented in Table 1. At any stage in the procedure the enzyme appeared to be quite stable for at least a week at 5°. However, 50% of the total activity of solution K (Table 1) was lost by freeze-drying the enzyme. No success has been realized as yet in crystallizing the enzyme. Electrophoretic analysis at pH 7·0 of an enzyme

extract containing 80 G.U./mg. P.N. (kindly carried out by Dr R. A. Kekwick of the Lister Institute) showed that at least two and probably three components with closely similar mobilities at this pH were present.

β-Glucuronidase activity of spleen extracts after incubation at 25° and dialysis

In the work described in the preceding sections it was observed that the β-glucuronidase activity of a spleen extract was increased by 5–7 hr. incubation at 25° followed by dialysis. Fig. 2 represents the results of an experiment designed to illustrate this point.

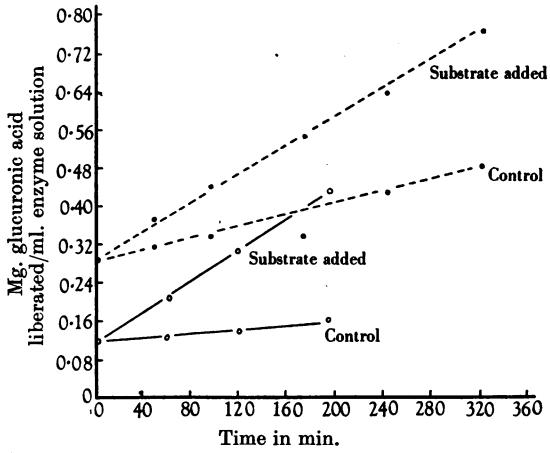

Fig. 2. Progress curves of extract of acetone-treated ox spleen. Broken lines: no preliminary treatment; solid lines: after incubation of extract for 8 hr. and dialysis.

An aqueous extract of spleen (A, Table 1) was divided into two portions. Time-action curves in the presence and absence of substrate at 25° and pH 5·0 were obtained for the first portion and are shown as broken lines in Fig. 2. A similar set of results was obtained with the second portion of extract after it had been incubated at 25° for 8 hr. and dialyzed overnight; this is represented by the solid lines in Fig. 2.

These curves show that the liberation of glucuronic acid from the substrate by incubated and dialyzed enzyme is about 55% more rapid than with the unincubated spleen extract. The first two columns of Table 2 show the activity in G.U./ml. extract for several different enzyme preparations before and after incubation for 6 hr. at 25°, followed by dialysis. Comparison of the figures in these columns shows that there is always a rise in activity after incubation and dialysis of the extract. Columns 3 and 4 indicate that there is an accompanying small decrease in P.N.

Table 2. *Effect of incubation at 25° for 6 hr. and dialysis of spleen extracts on β-glucuronidase activity and protein nitrogen*

	Activity (G.U.*/ml. enzyme solution)		Protein-N (mg./ml. enzyme solution)	
Preparation	Before incubation	After incubation and dialysis	Before incubation	After incubation and dialysis
25	0·130	0·200	2·20	1·54
26	0·190	0·295	2·23	1·54
27	0·060	0·140	—	—
28	0·100	0·150	—	—
29	0·120	0·170	—	—
30	0·095	0·120	—	—
31	0·140	0·180	—	—
33	0·100	0·140	1·43	1·11
34	0·055	0·115	2·16	1·81

* See p. 603.

To determine whether the increased activity of the extract after dialysis was due to the removal of an enzyme inhibitor formed during the previous incubation, in several experiments some of the dialysate was added to the enzyme and time-action curves were determined in the presence of substrate. Comparison of these results with those of a control experiment in which there was no added dialysate showed that there was no inhibition of the enzyme. Moreover, if an inhibitor were being formed during autolysis of the spleen extract, the time-action curve of the extract with added substrate would be expected to deviate from linearity. Inspection of the broken lines in Fig. 2 shows that this in fact does not occur during the period studied. It is hoped that work on the kinetics of the enzyme will provide an explanation for this curious observation.

Specificity of the enzyme

Masamune (1934) claimed that the enzyme prepared by his method was specific for the hydrolysis of β-glucuronides since there was no hydrolysis of menthol α-glucuronic acid, menthol β-glucoside, menthol α-glucoside and methyl α- and β-glucosides. Phenyl β-glucoside was hydrolyzed only to a slight extent. During the course of the present work several different enzyme preparations have been allowed to act on phenyl β-glucoside, salicin and cellobiose at 25° for 2 hr. at pH 5·0 and in no case has any hydrolysis of the glucoside been observed. It thus appears that the purified enzyme contains no β-glucosidase activity and is a specific glucuronidase.

DISCUSSION

Under the conditions of enzyme assay in the present work the final amount of glucuronic acid estimated in the cerimetric titration is seldom more than

100 μg. Since there is a simultaneous liberation of a much larger amount of reducing material by the initial ox-spleen extract independently of substrate hydrolysis, an error of considerable magnitude can occur in the estimation of β-glucuronidase activity. These findings serve to emphasize the inadequacy of an analytical method depending on the estimation of reducing power for determining enzyme activity in tissue extracts. Results obtained from such a non-specific method cannot be entirely free from suspicion unless the assay is properly controlled and the mechanism of the enzyme-catalyzed reaction is known. In the case of β-glucuronidase, unpublished results obtained by Dr G. A. Levvy in this Department show that for a freshly purified enzyme preparation the rate of substrate hydrolysis is the same whether measured colorimetrically by the Tollens reaction or by the cerimetric titration method. Masamune (1934) found that the rate of liberation of menthol was approximately the same as the rate of increase in reducing power and concluded that the chemical change effected in menthol glucuronide by the enzyme was confined to hydrolysis of the glucosidic link. It thus appears that, at least for the last three stages of purification as represented in Table 1, the figures for β-glucuronidase activity do represent the rate of liberation of glucuronic acid from the substrate.

Masamune (1934) reported that the maximum yield of β-glucuronidase from ox kidney was obtained after 72 hr. incubation at 38° of the tissue with twice its weight of water. This was later confirmed by Oshima (1936) for ox spleen and it appears from his figures that this procedure increased the yield of enzyme by at least 100%. The results reported by Oshima to illustrate this increase of activity were obtained on solutions which had been twice precipitated by ethanol after the autolysis period. It is not clear from Masamune's paper whether he introduced a similar stage of enzyme purification between autolysis and activity determination. Fishman (1939*b*), however, was unable to detect this increase in enzyme activity on autolysis of the spleen. In the present work it has been shown that autolysis of an ox-spleen extract for a few hours at 25° increases the β-glucuronidase activity in some cases by as much as 100%. The rise in activity, however, was observed only on dialysis of the autolyzed solution. It is therefore possible that dialysis of the autolyzed solutions in the present work was, in effect, equivalent to the precipitation of the enzyme with alcohol carried out by Oshima at the same stage, in so far as the β-glucuronidase activities of solutions prepared by either technique are higher than those of the original extracts. Fishman's inability to detect this rise was perhaps due to the fact that his assays were carried out directly on the spleen autolysates without preliminary dialysis or purification of the enzyme.

The procedure for purifying the enzyme described above seems to be somewhat more efficient than that of Fishman (1939*a*) in that the loss of total activity is generally about 50% compared to about 85% loss reported by him. Based on the activity of the initial spleen extract the purification effected by the present method is about 315 times, while Fishman reported about 140-fold purification. However, the fact that in the latter work, boiled enzyme controls were used in assaying the initial spleen extracts, and the error introduced by spontaneous liberation of reducing material from the enzyme solution was thus overlooked, suggests that Fishman's figure is incorrect and should be somewhat higher.

SUMMARY

1. A new method for the purification of ox-spleen β-glucuronidase has been devised. The method involves an isoelectric precipitation of inactive protein followed by ammonium sulphate fractionation at pH 4·3, 5·0 and 7·3.

2. The purified enzyme appears to be specific for the hydrolysis of β-glucuronides, since no hydrolysis of three common β-glucosides was observed in the presence of the enzyme.

3. Aqueous extracts of acetone-treated ox spleen when incubated at 25° at pH 5·0 liberate large amounts of dialyzable reducing material, the process being essentially complete after 6–8 hr. incubation.

4. The β-glucuronidase activity of aqueous extracts of acetone-treated ox spleen is increased, in some cases by 100%, by 6–8 hr. incubation at 25° followed by dialysis against water.

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