Purification of Cathepsin B from Oocytes and Mature Eggs of the Sea Urchin *Brissopsis lyrifera* by Means of Gel Filtration

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Cathepsin B from a homogenate fraction of *Brissopsis* oocytes has been purified 350 times by means of one gel filtration on Sephadex G-100. The chromatograms from oocytes and mature eggs differ in respect to highmolecular material, which, in the chromatogram from mature eggs is strongly reduced. The molecular weight of cathepsin B is estimated at a value of 50 000—60 000.

In the course of studies of proteolytic enzymes in the sea urchin egg at pH optima around 7 and using gelatin as substrate, an enzyme was also found that, according to the nomenclature of Fruton, Irving and Bergmann, belonged to the cathepsin II type. It had a pH-optimum at 4.5 and it split L-benzoylarginine amide in the presence of cysteine. Some years ago the catheptic enzymes were subdivided into the main types A, B, C, D, and E. In this new system cathepsin II is classified as cathepsin B. This enzyme was demonstrated both in unfertilized and fertilized sea urchin eggs. The highest activity was, however, recorded in oocytes of *Brissopsis lyrifera*. *Brissopsis* oocytes were therefore used as material for purification of cathepsin B. In the present investigation, gel filtration on Sephadex G-100 was chosen. The fractions were analyzed by means of UV absorption and tested for cathepsin B activity with gelatin as substrate. In two recent investigations the separation of proteolytic enzymes from sea urchin eggs was carried out by means of gel filtration and anion exchange on the crosslinked polysaccharide Sephadex. In *Brissopsis lyrifera* from the region of the Kristineberg Zoological Station on the Swedish west coast, the females were mainly found to contain only oocytes, a condition that is very rare among sea urchins. Practically all the eggs in one female mature simultaneously and spawning takes place very soon after maturation. This makes the collection of mature eggs rather fortuitous (see Runnström and Monné). However, one of the writers succeeded to find some females containing practically only mature eggs. This provided an opportunity of carrying out studies on the cathepsin B in the female gametes of *Brissopsis*, both before and after their maturation.

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MATERIAL AND METHODS

Material. The experiments on Brissopsis lyrifera were commenced at the Kristineberg Zoological Station in July 1962. Further investigations on the collected material were made at the National Bacteriological Laboratory, Stockholm. The oocytes of the mature eggs from the animals were filtered through bolting silk and washed by suspending them in sea water and removing the water after sedimentation. This was repeated three times. The material from each individual female was controlled under the microscope and the fertilization capacity of the batches of mature eggs was tested. The material was pooled separately, carefully centrifuged and the sedimented gametes were mixed with equal volumes of distilled water. For homogenization, the suspensions were sucked six times through a G 2 glass filter at +1°C (Runnström, Hagström and Löw). The cytolytases were checked under the microscope for complete cytolyysis. The two pools were divided into portions, which were frozen and kept at -25°C until use. Then a portion of the material was thawed and centrifuged at 15 000 × g for 20 min at +4°C. In most cases the supernatant was acidified with 0.5 M HCl to pH 4.5 and centrifuged again at 15 000 × g for 20 min at +4°C. The sediment from the first centrifugation was suspended in a certain volume of 0.05 M sodium citrate buffer at pH 4.5 containing 0.01 M cysteine, stirred in the cold for one hour, frozen to -80°C and kept at -25°C. Before use, a portion was thawed and centrifuged at 15 000 × g for 20 min at +4°C.

Fractionation. The chromatographic column was set up in the following manner. Portions of Sephadex G-100 (manufactured by AB Pharmacia, Uppsala, Sweden) were treated according to the instructions, equilibrated with the medium and poured into the column. The medium was buffered throughout with 0.02 M sodium citrate at pH 4.5. The effluent was collected in 3 ml fractions and the optical density was measured in a Beckman spectrophotometer, model DU, at 260 and 280 μm. The protein nitrogen in the applied samples was determined by means of the Kjeldahl micromethod. In the collected fractions after gel filtration the protein content was estimated according to a nomogram by Warburg and Christian.

Assay of proteolytic activity. The cathepsin B activity of the fractions was tested by its effect on the viscosity of a gelatin solution according to a method of Hultin as described in a previous paper. 0.5 ml of fraction and 0.5 ml 0.08 M cysteine were mixed with 3.00 ml of a 4.00 % gelatin solution in 0.1 M citrate buffer at pH 4.5 containing 0.01 % merthiolate as a sterilizing agent. The outflow times of the mixture were measured at 35.8°C in an Ostwald viscosimeter and the proteolytic activity calculated according to Hultin's formula. The value so obtained was multiplied by 10⁶ and called Hultin unit (H.U.).

RESULTS

The investigations on Brissopsis material were started with fresh homogenates from oocytes, which were centrifuged at 1000 × g for 15 min and filtered through a G-75 column (1.8 × 88 cm) in distilled water. Two peaks appeared, the cathepsin B activity measured at pH 4.5 being in the first peak. Sephadex G-100, which was then tested, gave a better separation and was therefore used in all subsequent studies.

An experiment with a fresh oocyte homogenate will be described here. The oocytes were homogenized in an equal volume of distilled water, centrifuged for 15 min at 12 000 × g, dialyzed for 65 h at +4°C against 0.02 M TRIS-HCl buffer at pH 7.4, and centrifuged for one hour at 12 000 × g in the cold. When the supernatant was applied to the column (Sephadex G-100 in 0.02 M Na-acetate, pH 4.5), a precipitate was formed at the top where it remained during the whole run. Three peaks were obtained and the fractions were tested for catheptic activity at pH 4.5. The activity expressed in H.U./ml

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Fig. 1. Gel filtration of a frozen and precipitated homogenate from *B. dissjosis* oocytes on Sephadex G-100. The homogenate was frozen and thawed twice, and then centrifuged, precipitated at pH 4.5 and centrifuged again as described in the text. Buffer 0.02 M Na citrate, pH 4.5. Column size, 1.6 × 160 cm. Sample applied, 3.5 ml. Volume of fractions, 3 ml. Rate of elution 9 ml/h. Temperature +5°C.

was in the order of 0, 1830, and 50. The main activity was thus in the second peak. The total activity of the applied sample was found to be 1780 H.U./ml.

In all experiments reported below, the material used had been frozen and thawed.

A good separation of cathepsin B from other components in the oocyte homogenate is shown in Fig. 1. The frozen homogenate was thawed and frozen twice before the treatment which included an acidification to pH 4.5 and separation of the formed precipitate by centrifugation. The supernatant was used for the gel filtration. Three well-separated peaks were obtained; a fourth peak at the end of the chromatogram was less distinct. The second peak contained no catheptic activity. The peak carrying the catheptic activity, contained as little as 20 µg protein per ml. The optimal activity of cathepsin B was 1140 H.U./ml in fraction 59, giving the activity of 57 000 H.U. per mg protein. The activity of the original sample was 2900 H.U./ml or 160 H.U./mg protein. Thus, a purification factor of 356 was attained by means of one single gel filtration.

The sediment from the homogenate used in Fig. 1 was extracted as described in the legend of Fig. 2 and was the subject of a gel filtration on G-100 in the same column. As seen in Fig. 2, the first peak is very low, the second
has almost disappeared and the third and the fourth peaks are considerably reduced. In comparison with the low absorbancy of the fractions, the catheptic activity is still conspicuous. The optimal activity in fraction 59 is 260 H.U./ml or 26,000 H.U. per mg protein. The activity of the sample was 2520 H.U./ml or 810 H.U. per mg protein. Thus the factor of purification is 32. Calculated upon the original activity of the sample used in the experiment shown in Fig. 1, the factor is 163. It may be observed that, in both these chromatograms, the optimal cathepsin B activity occurs in exactly the same tube, i.e. No. 59.

A homogenate from mature *Brissopsis* eggs was treated in the same way as the oocyte homogenate used in Fig. 1 and the result of a G-100 gel filtration is shown in Fig. 3. The first high peak appearing in the fraction interval 30—40 in Fig. 1 has almost disappeared and the highest absorbancy is found in fraction 31, being 0.508 at 280 m\(\mu\). The second peak does not diverge greatly from the corresponding peak in a chromatogram relating to oocytes. The third peak has a deviating shape and an appreciable amount of UV absorbing material is present in the interval between the second and the third peaks. The cathepsin B activity has its optimum in fraction 55, i.e. 660 H.U./ml or 8450 H.U. per mg protein. The activity of the applied sample was 2900 H.U./ml or 270 H.U. per mg protein, which gives a purification factor of 31 after gel filtration. The densities of the applied samples of oocytes and mature eggs were of the same order of magnitude but the optimal activity was only half that of the cathepsin B activity in fraction 59, Fig. 1.

The sediment from the mature *Brissopsis* egg homogenate was also extracted; the chromatogram had a pattern different from that recorded for oocyte material. Fig. 4 shows such a chromatogram from a frozen, thawed and centrifuged sediment extract. The first peak is almost missing as in Fig. 3 but between the second and the third peaks an extended interval of UV absorbing material can also be seen here. In chromatograms from oocyte

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Fig. 3. Gel filtration of a frozen and precipitated homogenate from mature *Brissopsis* eggs on Sephadex G-100. The material was treated as described in the text. Buffer 0.02 M Na-citrate pH 4.5. Column size 1.6 × 165 cm. Sample applied, 12 ml. Volume of fractions, 3 ml. Rate of elution 12 ml/h. Temperature +5°C.

Fig. 4. Gel filtration of an extract from *Brissopsis* egg on Sephadex G-100. The sediment from the first centrifugation (15 min at 15 000 × g) of the material used in experiment shown in Fig. 3 was suspended in 6 ml 0.05 M Na-citrate, pH 4.5 with 0.01 M cysteine and treated as described in the text. Buffer 0.02 M Na-citrate, pH 4.5. Column size, 1.6 × 165 cm. Sample applied, 5 ml. Volume of fractions, 3 ml. Rate of elution 6 ml/h. Temperature +5°C.

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homogenates as shown in Fig. 1, there are only minute amounts of UV absorbing material in the interval between the second and the third peaks. The optimal cathepsin B activity estimated in the material presented in Fig. 4 is 660 H.U./ml (fraction 64) or 12 000 H.U. per mg protein (fraction 63). The activity of the sample was 4700 H.U./ml or 2400 per mg protein, the low purification factor 5 being thus obtained in fraction 63. As only a small quantity of mature Brissopsis eggs were available the experiments shown in Figs. 3 and 4 were performed only once.

DISCUSSION

In comparison with most other well-known proteolytic enzymes, the cathepsins have only been purified to a limited extent. None of the cathepsins has been obtained in crystalline state, which is probably due to their complex form, i.e. the different types are not single enzymes but multiple molecular forms, isoenzymes.

By means of combined methods, Greenbaum and Fruton\textsuperscript{14} purified beef cathepsin B 190 times calculated per mg protein, and Lapresle and Webb\textsuperscript{15} purified cathepsin E from rabbit bone marrow using chromatography on DEAE cellulose and gel filtration on Sephadex G-75. They attained a purification factor of 22. Cathepsin D from bovine spleen has been purified 2-300 times by Press \textit{et al.}\textsuperscript{16}, employing a procedure involving salt precipitation, anion- and cation chromatography and starch-gel electrophoresis. Ten active components of cathepsin D were thereby separated.

In the present work, cathepsin B from oocyte homogenates, centrifuged at 15 000 $\times g$, has been purified 350 times by means of a single gel filtration on Sephadex G-100 as shown in Fig. 1. The high enzyme activity in the second peak in the experiment described above (G-100 in 0.02 M Na-acetate, pH 4.5) is equal to that of the applied sample. This is quite unusual as gel filtration involves a dilution of the sample. The sample in the earlier experiment was dialyzed and it is possible that an inhibitor had been removed. Activation of cathepsin B after dialysis has previously been reported by one of the writers\textsuperscript{4} and the presence of a cathepsin B inhibitor in the supernatant fraction from rat liver homogenate was demonstrated by Finkenstaedt\textsuperscript{17}.

In all other experiments the samples were undialyzed and we find in Fig. 1 that the activity per volume after the gel filtration is only 40 % of that of the sample used for gel filtration, and in Fig. 2 (the extracted sediment from the homogenate used in Fig. 1) the highest activity in any fraction is only 10 % compared with the sample. In Fig. 3, which shows a chromatogram from mature eggs, the optimal activity per volume was 25 % of that of the sample and in the chromatogram, Fig. 4, (referring to the extracted sediment from Fig. 3) it was 15 %.

The position of cathepsin B activity might indicate a molecular weight of 50 000—60 000. After gel filtration on Sephadex G-75, the cathepsin B was found in the first peak indicating a molecular weight greater than 50 000. Evtikhina and Chernikow\textsuperscript{18} have purified a cathepsin from beef spleen, splitting hemoglobin at pH 3. They determined the molecular weight to about 60 000.

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The extinction chromatogram from oocytes and from mature eggs are quite different. The first peak representing components with molecular weights greater than 100,000 is strongly reduced in the chromatogram from mature eggs, which, however, has a greater amount of components of lower molecular weights.

The question arises as to how the high-molecular material of the first peak shown in Fig. 1 is converted in connection with maturation. According to the optical densities measured at 280 and 260 μ, it contains nucleoproteins. These may be broken down during the maturation. Another explanation might be that the relative nuclear volume is much greater in oocytes that in mature eggs.19

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