On the distribution of *O*-seryl-*N*-acetylglactosaminide glycosidase and *N*-acetyl- β -*D*-hexosaminidase in vertebrates and invertebrates

HUBERTA SCHAUER and ALFRED GOTTSCHALK

Max-Planck-Institut für Virusforschung, Tübingen

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The enzymes *O*-seryl-*N*-acetylglactosaminide glycosidase and *N*-acetyl- β -*D*-hexosaminidase were found in the following invertebrates and vertebrates: *Adamsia palliata*, *Mesostoma ehrenbergi*, *Bugula avicularia*, *Lumbricus terrestris*, *Pagurus prideauxi*, *Helix pomatia*, *Ciona intestinalis*, *Bra-chidano albolineatus*, *Rana esculenta*, chicken and ox. They were assayed by their action on neuraminic acid-free ovine submaxillary glycoprotein, which contains an *O*-seryl-*N*-acetylglactosaminide linkage, and on phenyl *N*-acetyl- β -*D*-glucosaminide respectively. As previously shown for ox spleen and *Helix pomatia*, also the two enzyme proteins present in the homogenates of chick embryo and *Lumbricus terrestris* eluted from Sephadex columns in two separate peaks.

It was reported previously¹ that the supernatant of the homogenate of ox spleen contains two distinct hexosaminidases. One of them, the well established *N*-acetyl- β -*D*-hexosaminidase (EC 3.2.1.30.), splits off from oligosaccharides, prepared from hyaluronic acid and from chondroitin 4-sulphate by action of testicular hyaluronidase (EC 3.2.1.35) and β -*D*-glucuronidase (EC 3.2.1.31), *N*-acetylglucosamine and *N*-acetylglactosamine respectively from the non-reducing terminal. Its enzymic activity is conveniently assayed using phenyl or *p*-nitrophenyl *N*-acetyl- β -*D*-glucosaminide as substrate.

The other hexosaminidase acts on NANA-free OSM* and on glycopeptides prepared therefrom. In these compounds unsubstituted *N*-acetylglactosamine residues are linked *O*-glycosidically to peptide-bonded serine and threonine residues. The enzyme cleaving this glycosidic linkage was tentatively termed *O*-seryl-*N*-acetylglactosaminide glycosidase. The two enzyme proteins could be separated and purified by gel filtration^{1,2}. Highly purified *N*-acetyl- β -*D*-hexosaminidase is inactive towards NANA-free OSM and large glycopeptides prepared from it.

When it was found³ that the digestive juice of *Helix pomatia* also contains the above specified hexosaminidases, it was decided to make a systematic investigation of their distribution in the animal kingdom.

For the experiments the animals shown in Table 1 were used. Of the invertebrates *Mesostoma ehrenbergi* was a gift from Professor V. SCHWARTZ, Tübingen, the digestive juice of *Helix pomatia* was generously provided by l'Industrie Biologique Française, Gennevilliers (Seine), and *Lumbricus terrestris* was collected locally. The other invertebrates were gifts from the Stazione Zoologica at Naples and transported alive from there to Tübingen. The animals or specified organs of them were homogenized in a starmix (electrostar) for 2 min at 4°C in an appropriate volume of 0.1 M citrate buffer, *p*_H 4.8, containing 0.1 M NaCl. The homogenate was centrifuged at 15 000 *g* for 20 min at 4°C. The clear supernatant was used for assaying the enzymic activities. The substrates used in the assays were phenyl *N*-acetyl- β -*D*-glucosaminide and NANA-free OSM for β -acetylglucosaminidase and seryl-*N*-acetylglactosaminidase respectively.

The β -acetylglucosaminidase assay contained 0.5 ml of 0.02 M phenyl *N*-acetyl- β -*D*-glucosaminide in 0.1 M citrate buffer, *p*_H 4.4, 0.4 ml distilled water and 0.1 ml of the enzyme solution. Digestion at 37°C was carried out for 1 or 2 h. The phenol released was determined according to PUGH, LEABACK and WALKER⁴. The seryl-*N*-acetylglactosaminidase assay consisted of 0.2 ml aqueous solution of NANA-free OSM (see reference¹), containing 265 μ g ga-

¹ A. S. BHARGAVA, E. BUDDECKE, E. WERRIES, and A. GOTTSCHALK, Biochim. biophysica Acta [Amsterdam] **127**, 457 [1966].

* Abbreviations: OSM, ovine submaxillary gland glycoprotein; NANA, *N*-acetylneuraminic acid; β -acetyl-glucosaminidase, *N*-acetyl- β -*D*-hexosaminidase; seryl-*N*-acetylglactosaminidase, *O*-seryl-*N*-acetylglactosaminide glycosidase.

² E. BUDDECKE and E. WERRIES, Z. Naturforschg. **19b**, 798 [1964].

³ A. S. BHARGAVA and A. GOTTSCHALK, Biochem. biophysic. Res. Commun. **24**, 280 [1966].

⁴ D. PUGH, D. H. LEABACK, and P. G. WALKER, Biochem. J. **69**, 464 [1957].

Name of animal	<i>N</i> -Acetyl- β -D-hexosaminidase Protein per assay [mg]	Milliunits per assay	Specific activity [milliunits] (a)	<i>O</i> -Seryl- <i>N</i> -acetylgalactosaminidase Protein per assay [mg]	Milliunits per assay	Specific activity [milliunits] (b)	Ratio <i>a/b</i>
<i>Adamsia palliata</i>	0.145	207.0	1428	0.232	0.081	0.349	4092
<i>Mesostoma ehrenbergi</i>	0.179	16.6	92.7	0.268	0.207	0.772	120.1
<i>Bugula avicularia</i>	0.113	181.0	1602	0.252	0.275	1.091	1468
<i>Lumbricus terrestris</i>	0.584	115.0	196.9	1.168	0.242	0.207	951.2
<i>Helix pomatia</i> (digestive juice)	0.080	14.5	181.2	0.240	0.152	0.633	286.3
<i>Pagurus prideauxi</i>	0.372	356.0	957.0	0.372	0.115	0.309	309.7
<i>Ciona intestinalis</i>	0.102	334.0	3275	0.186	0.155	0.833	3932
<i>Brachidanio albolineatus</i>	0.243	8.5	35.0	0.486	0.030	0.062	564.5
<i>Rana esculenta</i> (oviduct)	0.050	8.3	166.0	0.500	0.238	0.476	348.7
Chick embryo	0.117	5.3	45.3	0.293	0.024	0.082	552.4
Ox (spleen)	—	—	10.0	—	—	~ 0.05	~ 200

Table 1. Presence of *N*-acetyl- β -D-hexosaminidase and *O*-Seryl-*N*-acetylgalactosaminidase in invertebrates and vertebrates.

lactosamine (expressed as galactosamine hydrochloride), and 0.2 ml of the enzyme solution (p_H 4.8). The digestion at 37° in the presence of toluene proceeded for 20 to 40 h. Suitable enzyme dilutions and digestion periods were found out in a preliminary experiment. With each assay a substrate and an enzyme control were run. The results are shown in Table 1. In Table 2 the phylogenetic classification⁵ of the animals tested is given.

Phylum	Sub-group or class	Animal
Coelenterata	Anthozoa	<i>Adamsia palliata</i>
Parenchymia	Plathelminthes	<i>Mesostoma ehrenbergi</i>
Tentaculata **	Bryozoa	<i>Bugula avicularia</i>
Articulata	Annelida	<i>Lumbricus terrestris</i>
Articulata	Arthropoda	<i>Pagurus prideauxi</i>
Mollusca	Gastropoda	<i>Helix pomatia</i>
Chordata	Tunicata	<i>Ciona intestinalis</i>
Chordata	Vertebrata	<i>Brachidanio albolineatus</i> (Pisces)
	Vertebrata	<i>Rana esculenta</i> (Amphibia)
	Vertebrata	Chicken (white leghorn) (Aves)
	Vertebrata	Ox (Mammalia)

Table 2. Phylogenetic classification * of the animals shown in Table 1. * The classification is based on A. KÜHN's Grundriß der Allgemeinen Zoologie, 1961. ** One of the smaller Protostomia groups.

The figures in Table 1 refer to the supernatant of the homogenates. In case of *Lumbricus terrestris* about one-third of the specific activity of seryl-*N*-

acetylgalactosaminidase and about 40% of the specific activity of β -acetylglucosaminidase were found in the precipitate (see also CONCHIE and HAY⁶).

Ten ml of the crude enzyme preparation of chick embryo were applied to a Sephadex G-150 column (150 cm $\times$ 1.6 cm) and fractionated (2 ml fractions) as described previously¹. The maximum of β -acetylglucosaminidase activity was in fraction 17 (specific activity = 555 milliunits) and that of seryl-*N*-acetylgalactosaminidase activity in fraction 31 (specific activity = 0.373 milliunits).

Three ml of the crude enzyme preparation of *Lumbricus terrestris* were applied to a Sephadex G-150 column (100 cm $\times$ 1.8 cm) and fractionated as above. The maximum of seryl-*N*-acetylgalactosaminidase activity was in fraction 24 (specific activity = 9.25 milliunits) and that of β -acetylglucosaminidase in fraction 30 (specific activity = 1690 milliunits).

From the results presented it is evident that β -acetylglucosaminidase and seryl-*N*-acetylgalactosaminidase are present in the metazoa from Coelenterata to Mammals. The specific activity of the former enzyme is invariably several hundred or even a few thousand times higher than that of the latter. As previously shown for ox spleen and *Helix pomatia*, also the two enzyme proteins present in the homogenates of chick embryo and *Lumbricus terrestris* eluted from Sephadex columns in two sepa-

⁵ See A. KÜHN, Grundriß der Allgemeinen Zoologie, 14. Aufl. Georg Thieme Verlag, Stuttgart 1961.

⁶ J. CONCHIE and A. J. HAY, Biochem. J. **87**, 354 [1963].

rate peaks. It may be pointed out that the specific activities of the two enzymes recorded in Table 1 for various animals cannot be compared with each other since the supernatants of the homogenates contain only a portion of the total content of the respective hexosaminidases and since the instability of seryl-*N*-acetylgalactosaminidase³ may vary with the source. The relatively high specific activity of seryl-*N*-acetylgalactosaminidase of the oviduct of *Rana*

esculenta was observed only in frogs shortly before spawning.

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Some Immunochemical Characteristics of Sea Urchin Gametes

STJEPAN KEČKEŠ¹ and MIRJANA KRAJNOVIĆ

Institute „Ruder Bošković“ and Institute of Marine Biology, Rovinj and Zagreb, Yugoslavia

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By double diffusion in agar the antigenic composition of four sea urchin species was examined. At least one common antigenic determinant was found in both gametes of all the four species investigated. Furthermore, in all eggs one and in all sperm another common “sex specific” antigenic determinant was established. In spite of the great number of antigenic determinants in each gamete extract, the existence of “species specific” determinants, common to sperm and eggs of one species, could not be determined with certainty.

Fertilization is at least partly due to the specific interaction between the chemical constituents of the sperm and egg surface. There are several methods for studying the chemical characteristics of the gametes, especially those which could play a certain role in fertilization. One of the best method is the immunochemical analysis introduced by TYLER². This method, which with regard to specificity is far beyond the classical chemical analyses, uses the very sensitive and highly specific antigen-antibody reaction to discover the chemical composition of biological material.

Using the immunochemical analyses PERLMANN³ found that in gamete extracts of *Paracentrotus lividus*, a common Mediterranean sea urchin, a great number of antigenically strongly different components are present. Furthermore, he showed that some of these components have a definite and specific effect when combined with the corresponding antisera. KÖHLER and METZ⁴ found a great number of different antigens in sperm extracts of various sea urchins and some of them proved to be associated to the sperm surface⁵.

It seemed worthwhile to continue these investigations and compare the results obtained with those of the investigations on homologous and heterologous fertilization⁶⁻⁸.

Material and Methods

Gametes of four different sea urchin species, common along the west Istrian coast of the Adriatic sea (*Paracentrotus lividus* Lam., *Arbacia lixula* L., *Sphaerechinus granularis* Lam. and *Psammechinus microtuberculatus* Biv.) were used.

Freshly caught animals were opened by a circular cut with scissors and the ripe gonads were removed, separated according to sex and species, cut in smaller pieces and placed over dense gauze in a plastic funnel. The extruded gametes were collected in bottles, cooled in ice and used freshly or freeze-dried.

Antisera were obtained by hyperimmunization of rabbits with fresh or freeze-dried gametes mixed with adjuvant⁹. Ten days after the last injection of antigen the blood of rabbits was collected, sera were separated by centrifugation and stored at -20°C until use.

The following symbols were used for the antigens and the corresponding antisera:

¹ Present address: Laboratory of Marine Radioactivity, International Atomic Energy Agency, Monaco.

² A. TYLER, *Physiol. Rev.* **28**, 180 [1948].

³ P. PERLMANN, *Experientia* [Basel] **15**, 41 [1959].

⁴ K. KÖHLER and C. B. METZ, *Biol. Bull.* **118**, 96 [1960].

⁵ G. P. FLAKE and C. B. METZ, *Biol. Bull.* **123**, 472 [1962].

⁶ B. E. HAGSTRÖM and S. LÖNNING, *Sarsia* **4**, 5 [1961].

⁷ S. KEČKEŠ, Ph. D. thesis, Univ. of Zagreb 1962.

⁸ B. E. HAGSTRÖM, *Sarsia* **17**, 33 [1964].

⁹ J. FREUND and K. McDERMOTT, *Proc. Soc. exp. Biol. Med.* **49**, 548 [1942].