Peptide:N-Glycosidase Activity Found in the Early Embryos of Oryzias latipes (Medaka Fish)

THE FIRST DEMONSTRATION OF THE OCCURRENCE OF PEPTIDE:N-GLYCOSIDASE IN ANIMAL CELLS AND ITS IMPLICATION FOR THE PRESENCE OF A DE-N-GLYCOSYLATION SYSTEM IN LIVING ORGANISMS*

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The recent discovery of free oligosaccharides typical for the complex type of glycan chains terminating with a free di-N-acetylated glycoprotein structure in certain fish eggs and early embryos (Ishii, K., Iwasa, M., Inoue, S., Kenny, P. T. M., Komura, H., and Inoue, Y. (1989) J. Biol. Chem. 264, 1623–1630; Seko, A., Kitajima, K., Iwasa, M., Inoue, S., and Inoue, Y. (1989) J. Biol. Chem. 264, 15922–15929; Inoue, S., Iwasa, M., Ishii, K., Kitajima, K., and Inoue, Y. (1989) J. Biol. Chem. 264, 18520–18526) led us to find an enzyme responsible for detachment of N-linked glycan chains from glycoproteins by hydrolyzing the β-asparaginyl-glucosamine linkage in Oryzias latipes embryos. The enzyme, peptide-N4-(N-acetyl-β-glucosaminy1) asparagine amidase or peptide:N-glycosidase (PNGase), was partially (2090-fold) purified, and the reaction site at which this enzyme acts was specified by analysis and identification of the reaction products. This is the first demonstration showing PNGase in animal sources, although the presence of PNGases was reported in a variety of plant extracts and bacteria. Thus, the commonality of this type of enzyme is now demonstrated, and the possible physiological role of PNGase in de-N-glycosylation as a basic biologic process is proposed.

In eukaryotic cells, the biosynthetic concept of N-glycosylation has been well documented. However, the necessity of removal of specific N-glycan chain(s), if any, from certain glycoproteins, which is possibly a prerequisite for intracellular transport, proteolysis, and other unknown functions, remains undiscovered in any system. During the course of our structural and functional studies on glycoproteins found in fish oocytes and embryos, we found that free N-glycan chains accumulated in two different developmental stages of fish embryos. The first example is the accumulation of free sialooligosaccharides in the unfertilized eggs of a fresh water trout (Plecoglossus altivelis) (1) and a dace (Tribolodon hakonensis) (2). All of these free sialooligosaccharides were typical complex types of bi-, tri-, and tetra-antennary chains. Relatively large amounts of free oligosaccharides accumulated in mature fish eggs, suggesting that they may possibly be derived from vitellogenins, abundant glycolipoprophoproteins incorporated and processed by the oocytes during oogenesis. As the second example, we have shown the presence of free penta-antennary glycan chains in the embryos (4–11 h postinsemination) of a flounder (Paralichthys olivaceus) (3). In this case, the free glycan was derived from the unique penta-antennary glycan structure of the cortical alveolar glycoprotein (hyosophorin) of this fish. Because the hyosophorin-derived free oligosaccharide is not found in unfertilized eggs or inseminated eggs that fail to continue embryonic development, de-N-glycosylation appears to have some biological significance during embryogenesis of the fish. The most important common structural feature of the free oligosaccharides found in fish eggs is the presence of a di-N-acetylated glycoprotein (PNAc) at their reducing termini. This fact indicates that 1) these oligosaccharides are detached from glycoproteins and 2) the enzyme catalyzing the scission must be peptide-N4-(N-acetyl-β-glucosaminy1) asparagine amidase or peptide:N-glycosidase (PNGase).¹

In this study, we demonstrate the presence of PNGase activity in the embryos of Medaka fish (Oryzias latipes) by partial purification of the enzyme and the structural analysis of the reaction products. PNGases have so far been obtained from almond emulsin (4), Flavobacterium meningosepticum (5), and eight plant seeds (6), but never from animal sources. Our present findings are thus important as the first indication of the presence of PNGase in animal cells and may suggest the importance of a protein N-glycosylation and de-N-glycosylation system in the metabolism and possibly the function of glycoproteins.

EXPERIMENTAL PROCEDURES

Material—About 1000 Medaka fish were purchased from the farm of cultivated stocks in Yatomi near Nagoya, and fertilized eggs (embryos at blastulae stage) were collected every morning and stored at −80 °C until use. Medaka t-hyosophorin (cortical alveolus-derived glycononapeptide, Asp-Ala-Ala-Ser-Asn(CH0)-Gin-Thr-Val-Ser) was isolated from the fertilized eggs (7). Fetuin glycophosphate (Leu-Ala-Asn(CH0)-CmCys-Ser) was prepared from fetal calf serum fetuin (Nacalai Tesque, Kyoto) by the methods of Plummer et al. (6).

Radiolabeling of t-Hyosophorin and Fetuin Glycopeptide by Reductive Methylation—Dried sample (1.6 μmol) was dissolved in 800 μl of 0.2 M sodium phosphate buffer (pH 7.1) and 100 μl of pyridine borane in methanol (16.2 μl/ml), and 100 μl of 3.1 MBq H⁺CHO in water (Du Pont/New England Nuclear, 2.2 GBq/mmol) were added

¹ The abbreviations used are: PNGase, peptide:N-glycosidase; CmCys, carboxymethylcysteine.

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to the solution (8). The reaction was continued for 4 h at room temperature. After additional reaction with 16.2 μl of pyridine borane and 20 μl of 3.7% cold formaldehyde at room temperature for 2 h, the mixture was applied to a Sephadex G-50 column (superfine, 1.2 × 72 cm; equilibrated and eluted with 0.1 M NaCl). The peak fractions were pooled and desalted by passage through a Sephadex G-25 column. Incorporation of radiolabeled methyl groups was 0.59 mol/mol in fetuin glycopeptide II, \((\text{C}^1\text{C})\text{Me}-\text{NH}-\text{Leu-Ala-Asn}-(\text{CHO})-\text{CmCySer-COO}^-, \) and 0.69 mol/mol in Medaka L-hyosophorin, \((\text{C}^1\text{C})\text{Me}-\text{NH}-\text{Asp-Ala-Asn}-(\text{CHO})-\text{Gln-Thr-Val-Ser-COO}^-\).

Chemical Analysis—Methods of carbohydrate analysis were described previously (9). Amino acid analysis was carried out with hydrolases in 6 N HCl at 110 °C for 24 h under N2 and precolumn derivatization with phenylisothiocyanate (10). Automated peptide sequence analysis was performed on an Applied Biosystem model 477A protein sequencer (3).

Assay of PNGase Activity.—The enzyme solution (6 μl) containing 10-20 mM sodium acetate (pH 6.0), 0.25 mM sucrose, 0.6 μg of soybean trypsin inhibitor (Sigma) and L-\([^{14}\text{C}]\)hydroxyinosine (30,000 cpm) was incubated in a polypropylene microtube for 16 h at 25 °C. The reaction mixture was clarified by centrifugation and applied as a spot on Whatman 3-MM paper. The chromatogram was developed ascendingly in butanol/ethanol/water (4:2:3 v/v) at room temperature (11) until the solvent front migrated up to 7.5 cm. The dried chromatogram was cut in horizontal sections for the measurement of radioactivity. Under these conditions, \([^{14}\text{C}]\)-peptide released from L-\([^{14}\text{C}]\)hydroxyinosine incubated with PNGase F (Takara Shuzo, Co., Kyoto) migrated 1.2-1.8 cm from the origin. However, intact glycopeptide II remained at the origin.

PNGase activity in fish egg preparations was determined from the radioactivity found in the area of paper chromatogram between 1.2 and 1.8 cm from the origin. One unit was defined as the amount of the enzyme releasing 1 nmol of \([^{14}\text{C}]\)-peptide from L-\([^{14}\text{C}]\)hydroxyinosine upon incubation for 16 h at 25 °C under the above-described conditions.

Partial Purification of PNGase from Medaka Embryos—All purification procedures were done at 4 °C. 233 g of Medaka embryos were homogenized in 370 ml of sodium acetate buffer (pH 6.0) containing 0.25 mM sucrose, 3 mM EDTA, and 0.1 mg/ml soybean trypsin inhibitor with a Waring blender and filtered through Tetoron gauze. The filtrate was centrifuged first at 6,000 × g for 1 h, and the supernatant was dialyzed against 10 mM sodium acetate buffer (pH 6.0), 0.25 mM sucrose. The dialyzed solution was applied on a CM-Sephadex C-25 column (1.75 × 31 cm) equilibrated with 10 mM sodium acetate buffer (pH 6.0), 0.25 mM sucrose. To the breakthrough fraction (836 ml) was slowly added 1090 ml of saturated ammonium sulfate solution (pH 7.1), and the mixture was left overnight. The precipitate collected by centrifugation at 20,000 × g for 30 min was dissolved in 10 ml of 10 mM Tris-HCl (pH 8.0), 0.25 mM sucrose, diazylated against the same solution, subsequently applied on a Whatman DE-52 column (2.0 × 39 cm, equilibrated with 10 mM Tris-HCl (pH 8.0), 0.25 mM sucrose), and eluted with a linear gradient (0-0.3 M) of NaCl in the equilibration buffer. The fraction containing PNGase activity was pooled and purified through a series of chromatography on columns of Whatman DE-52 (1.7 × 23 cm), DEAE-Toyopearl (Toyo, Co., Tokyo, 17 × 25 cm), Sepharly S-200 (1.5 × 68 cm, equilibrated and eluted with 10 mM sodium acetate buffer (pH 6.0), 0.25 mM sucrose). To the breakthrough fraction (836 ml) was slowly added 1090 ml of saturated ammonium sulfate solution (pH 7.1), and the mixture was left overnight. The precipitate collected by centrifugation at 20,000 × g for 30 min was dissolved in 10 ml of 10 mM Tris-HCl (pH 8.0), 0.25 mM sucrose, diazylated against the same solution, subsequently applied on a Whatman DE-52 column (2.0 × 39 cm, equilibrated with 10 mM Tris-HCl (pH 8.0), 0.25 mM sucrose), and eluted with a linear gradient (0-0.3 M) of NaCl in the equilibration buffer. The fraction containing PNGase activity was pooled and purified through a series of chromatography on columns of Whatman DE-52 (1.7 × 23 cm), DEAE-Toyopearl (Toyo, Co., Tokyo, 17 × 25 cm), Sepharly S-200 (1.5 × 68 cm, equilibrated and eluted with 10 mM sodium acetate buffer (pH 6.0), 0.25 mM sucrose, 5 mM EDTA, 0.15 M NaCl), Whafman DE-52 (1.0 × 20 cm), and DEAE-Toyopearl (1.2 × 17.5 cm). All anion-exchange columns were equilibrated with 10 mM Tris-HCl (pH 8.0), 0.25 mM sucrose.

Digestion of Fetuin Glycopeptide II and L-Hyosophorin with Partially Purified Medaka Embryo PNGase and Identification of the Products—A 40-nmol sample of \([^{14}\text{C}]\)-fetuin glycopeptide or L-hyosophorin was incubated with a PNGase sample (18.4 milliunits) dissolved in 0.4 ml of 20 mM sodium acetate buffer (pH 6.0) containing 0.25 mM sucrose, 50 mM NaCl, 5 mM EDTA, and 40 μg of soybean trypsin inhibitor at 25 °C for 48 h under N2. Another three 0.4-nmol portions of the enzyme were added at 12-h intervals. The reaction mixture was applied to a Sephadex G-50 column (1.2 × 72 cm, eluted with 0.25 M sucrose, 50 mM NaCl, 5 mM EDTA, and 40 μg of soybean trypsin inhibitor at 25 °C for 48 h under N2). The released radiopeptide eluted in peak 2 (Fig. 3) was further purified on a Bio-Gel P-2 column (1.2 × 53 cm, eluted with 10 mM acetic acid), desalted on a Sephadex G-25 column, and subjected to composition and sequence analyses.

For the analysis of the released oligosaccharide from fetuin glycopeptide II, the material eluted in peak 1 from the Sephadex G-50 column (Fig. 3) was desalted on a Sephadex G-25 column and digested with 23 milliunits of Arthrobacter ureafaciens sialidase (Nacalai Tesque, Kyoto) under toluene in 0.5 ml of 50 mM sodium acetate buffer (pH 5.5) for 21 h at 37 °C. The reaction mixture was applied to a Sephadex G-50 column, and the fraction containing the free asialooligosaccharide and the asialoglycopeptidase was desalted by passage through a Sephadex G-25 column. Separation of the asialooligosaccharide and the asialoglycopeptide was affected by anion-exchange chromatography on a Bio-Rad AG 1-X2 column (0.5 × 6 cm, Cl- form). The asialooligosaccharide was eluted as a breakthrough peak. One third of the asialooligosaccharide was reduced with 2 mg of NaBH4 in 0.5 ml of 50 mM sodium borate buffer (pH 9.4) for 17 h at 25 °C before composition analysis.

For amino acid sequence analysis, 70 nmol of unmodified fetuin glycopeptide was digested with the PNGase fraction, and the released peptide was purified by a method similar to that described above for radiolabeled fetuin glycopeptide.

RESULTS AND DISCUSSION Detection of PNGase Activity in the Fertilized Medaka Eggs and Partial Purification of the Enzyme—We have found the presence of two types of free sialooligosaccharide chains in the Medaka embryos (12). The results of structural analysis of the free oligosaccharides indicated that one type is derived from hyosophorin and the other is from phosvitin (13). The fact that these oligosaccharides retain the di-N-acetylchitobiosyl structure at their reducing ends was considered to be an indication of the presence of PNGase in Medaka embryos. In the present work, we could detect in the extract of Medaka embryos the enzyme activity that catalyzes the release of N-glycan chains from glycopeptides. The enzyme in the soluble fraction of the extract was partially purified by ammonium sulfate precipitation and a series of anion-exchange column chromatography (Figs. 1 and 2), and the purification scheme is summarized in Table I. Ammonium sulfate precipitation was effective in separating the enzyme protein from endogenous glycopeptides, and repeated anion-exchange chromatography was necessary to remove yolk proteins that are abundant in fish embryos. An overall 2090-fold purification (specific activity, 1.57 units/mg of protein) and about 6% yield of PNGase was obtained from the crude extract of Medaka embryos. As judged from the results shown in Fig. 2, the PNGase fraction is still contaminated with proteinaceous material, and our preparation of the enzyme was not yet biochemically homogeneous. Nevertheless, the present study resulted in a 2090-fold purification with a 5.8% yield of activity, and the present PNGase is the first

2. A. Seko, unpublished observation.
animal-derived enzyme that has been identified and purified to this degree of purity.

Digestion of Fettuin Glycopeptide II with Medaka Embryo PNGase—Fettuin glycopeptide II was digested with partially purified Medaka PNGase, and the products were separated on a Sephadex G-50 column (Fig. 3a). High percentages of radioactivity (88%) were eluted in more retarded fractions (peak 2) than the intact glycopeptide (peak 1). The released peptide was recovered from peak 2, purified by Bio-Gel P-2 chromatography, and desalted on Sephadex G-25. Peak 1 contained the unreacted glycopeptide and the released asialoligosaccharide. To separate the oligosaccharide from the intact glycopeptide, this fraction was pooled as described under “Experimental Procedures,” and indicated by the bar.

Medaka embryo PNGase was identical with that of the fetuin glycopeptide II, except for the disappearance in the chromatogram of NH₂-terminal leucine that had been N,N-dimethylated. No glucoseamine was detected in the hydrolysate of the peptide fraction. The ratios of Gal and GlcNAc to 3-Man residues in the oligosaccharide fraction were identical with those in the parent glycopeptide. The reduction of the oligosaccharide resulted in conversion of 1 mol of GlcNAc to GlcNACol. No amino acid was detected in the hydrolysate of the oligosaccharide fraction. These results clearly indicate that oligosaccharide with di-N-acetylchitobiose at the reducing end was released by the enzyme. The amino acid sequence of fetuin glycopeptide II was determined to be Leu-Ala-Asn(CH0)-Cys-Cys-Ser. The amino acid sequence of the released peptide was Leu-Ala-Asn(Cys)-Cys-Ser (Table III). Note that the third glycosylated Asn was converted into an Asp residue after de-N-glycosylation with the Medaka enzyme. All of these results support identification of the enzyme activity derived from Medaka embryos as PNGase.

Digestion of [14C]Hyosophorin, ([14C]Me)₂NH⁺-Asp-Ala-Ala-Ser-Ala-Asn(CH0)-Gln-Thr-Val-Ser-COO⁻, with Medaka Embryo PNGase—After L-[14C]hyosophorin was incubated with the PNGase fraction from Medaka embryos, the released peptide, i.e., ([14C]Me)₂NH⁺-Asp-Ala-Ala-Ser-Ala-Asn(CH0)-Gln-Thr-Val-Ser-COO⁻, was separated from the parent glycopeptide on Sephadex G-50 (Fig. 3b, peak 2), purified on Bio-Gel P-2, and analyzed for amino acid and amino sugars after hydrolysis. As shown in Table II, amino acid composition of the released peptide was identical with that of the parent hyosophorin, except for the modified NH₂-terminal aspartic acid residue. No glucoseamine was detected in the hydrolysate. The results show that the N-glycan chain of L-hyosophorin is indeed released by the action of the PNGase fraction from Medaka embryos, and thus the accumulation of free N-glycan chains in Medaka and most probably in certain other fish embryos can be ascribed to the presence of this type of enzyme activity.

General Discussion—To our knowledge, no report for PNGase from an animal source has so far been found, although PNGase activity has been detected in the extracts of

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**Fig. 2. Anion-exchange chromatography on a DEAE-Toyopearl column (Cl⁻ form) of the PNGase-active fraction derived from the Medaka embryos.** The PNGase fraction, obtained as in Fig. 1 after rechromatography on the same Whatman DE-52 column followed by a series of chromatographic procedures as described under “Experimental Procedures,” was applied to a DEAE-Toyopearl column (Cl⁻ form) of the PNGase-active fraction derived from the Medaka embryos. The column (1.2 × 17.5 cm) was eluted with 0.1 M NaCl. Fractions (1.13 ml) were collected, monitored by radioactivity (expressed as cpm), and pooled as indicated with the bars. Two discrete radioactive fractions were labeled peak 1 and peak 2. a, peak 1, ([14C]Me)₂Leu-Ala-Asn(CH0)-Cys-Ser; peak 2, ([14C]Me)₂Leu-Ala-Asp-Cys-Ser; b, peak 1, ([14C]Me)₂Asp-Ala-Asn(CH0)-Gln-Thr-Val-Ser; peak 2, ([14C]Me)₂Asp-Ala-Asp-Gln-Thr-Val-Ser. Free glycans chains liberated by the action of PNGase were eluted under peak 1 in both a and b.

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**Fig. 3. Gel filtration on a Sephadex G-50 column of the digests of (a) [14C]-labeled fetuin glycopeptide II and (b) [14C]-labeled L-hyosophorin with a partially purified PNGase fraction obtained from the Medaka embryos.** The column (1.2 × 72 cm) was eluted with 0.1 m NaCl. Fractions (1.13 ml) were collected, monitored by radioactivity (expressed as cpm), and pooled as indicated with the bars. Two discrete radioactive fractions were labeled peak 1 and peak 2. a, peak 1, ([14C]Me)₂Leu-Ala-Asn(CH0)-Cys-Ser; peak 2, ([14C]Me)₂Leu-Ala-Asp-Cys-Ser; b, peak 1, ([14C]Me)₂Asp-Ala-Asn(CH0)-Gln-Thr-Val-Ser; peak 2, ([14C]Me)₂Asp-Ala-Asp-Gln-Thr-Val-Ser. Free glycans chains liberated by the action of PNGase were eluted under peak 1 in both a and b.

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**Table I**

| Fraction          | Activity* | Protein* | Specific Activity | Yield | Purification |
|-------------------|-----------|----------|------------------|-------|--------------|
| Crude extract     | 4.98      | 6630     | 0.000751         | 100   | 1            |
| (NH₄)₂SO₄ precip- | 2.55      | 176      | 0.0145           | 51    | 19           |
|itate             |           |          |                  |       |              |
| Second DE-52      | 1.71      | 71.4     | 0.0799           | 34    | 106          |
| Sephacryl S-200   | 1.32      | 3.82     | 0.346            | 27    | 461          |
| Second Toyopearl  | 0.288     | 0.183    | 1.57             | 5.8   | 2090         |

* Enzyme activity was determined using L-[14C]hyosophorin, and one unit was also defined as described under “Experimental Procedures.”

* Protein was quantified by the method of Lowry et al. (14).
since they are transported to lysosomes immediately after protein-derived free oligosaccharides would not be observed. N-acetylglucosaminidase do not retain a di-N-acetylchitobiosyl structure at their reducing termini have strongly supported the supposition that PNGase activity appears stage-specifically during oogenesis and/or embryogenesis and to clarify the significance of de-N-glycosylation of phosvitin and hyosophorin in the fish embryos. Recently, several reports have appeared that suggest that deglycosylation is a unique post-translational modification system for glycoproteins operating in vivo in certain plant cells. Concanavalin A, wheat germ agglutinin, β-1,3-glucanase, barley lectin, and rice lectin are all synthesized as N-linked glycoproteins and matured by post-translational processing of the glycopeptides (17–19). The two-step processing, i.e., removal of first N-linked glycan(s) and, second, proppeptide, was proposed for concanavalin A and barley lectin (19, 20). The removal of glycan chains was shown to affect the transport and maturation of the proppeptides (17, 19). In this connection, in the amino acid sequence of ricin, *Ricinus communis* seed lectin, as determined by protein sequencing, the 236th amino acid residue of the A unit was indicated as Asp (21), whereas this residue was deduced from cDNA base sequencing as Asn (22). It has been reported that a “heavy” or “variant” form of ricin A chain contains two N-glycan chains at Aan-10 and Aan-236 (23). The structure of the N-glycan chain at Aan-236 has recently been reported (24). If we combine these results, a “light” form of ricin A chain is most likely formed from the heavy form by site-specific de-N-glycosylation catalyzed by PNGase, which converts the asparagine glycosylamine bond at position 236 to aspartic acid. Our limited knowledge of de-N-glycosylation of N-linked glycoproteins catalyzed by PNGase prevents any further considerations about the functional role of de-N-glycosylation and of the free glycans liberated. Nevertheless, de-N-glycosylation of a glycopeptide or glycoprotein, which converts the carbohydrate-attached Asn residue to the Asp, thereby introducing negative charge and altering the peptide or protein conformation, may be a possible means to produce a functional conformation. To assess the precise functional role of the detachment of a large N-glycan chain from L-hyosophorin in certain fish species of free N-glycan the late stage of oogenesis (1, 2) and the blastulation stage of embryogenesis (3)* of certain fish species.

### TABLE II

| Peptide:N-Glycosidase in Fish Embryos |
|--------------------------------------|

#### TABLE III

*In vivo* digestion of fetuin glycopeptide II and the peptide obtained by digestion of fetuin glycopeptide II with the Medaka embryo PNGase

No PTH-derivatives were identified at cycle 3 (marked by X) of the fetuin glycopeptide II because this position is occupied by the glycosylated Asn residue as judged from the amino acid composition analysis. The yields of PTH-derivatives are expressed in picomoles of PTH-derivatives determined by high pressure liquid chromatography when 1 nmol each of the two samples was applied.

| Amino acid sequence determination of fetuin glycopeptide II and the peptide obtained by digestion of fetuin glycopeptide II with the Medaka embryo PNGase |
|-----------------------------------------------|

#### TABLE III

| Cycle | Fetuin glycopeptide II | Fetuin peptide |
|-------|------------------------|----------------|
|       | PMOL | PTH-derivatives | Carry-over | PMOL | PTH-derivatives | Carry-over |
| 1     | Leu (126) | Leu (116) |         |       |       |         |
| 2     | Ala (66)  | Ala (14)  | Ala (95) | Ala (28) |       |         |
| 3     | X         | Ala (13)  | Asp (23) | Ala (24) |       |         |
| 4     | CmCys (30)| CmCys (67)| Asp (19) |         |       |         |
| 5     | Ser (26)  | CmCys (10)| Ser (44) | CmCys (20) |       |         |
| 6     | Ser (12)  | Ser (16)  |         |         |       |         |

*ND, not done.*

### Table

| Table | Before digestion | After digestion |
|-------|------------------|----------------|
|       | 14-C-Peptide     | Asialooligosaccharide | Reduced asialooligosaccharide |
| Asp   | 1.2              | 1.1             | ND                          |
| Gla   | —                | —               | ND                          |
| CmCys | 1.0              | 0.92            | ND                          |
| Ser   | 1.0*            | 1.0*            | ND                          |
| Thr   | —                | —               | ND                          |
| Ala   | 1.0              | 1.1             | ND                          |
| Val   | —                | —               | ND                          |
| Leu   | 1.0              | —               | ND                          |
| Man   | 3.0              | ND*             | 3.0*                        |
| Gal   | 2.9              | ND              | 3.2                         |
| GlcNAc| 5.0              | 4.9             | 5.8                         |
| GlcNAcol | ND            | 0.38            | ND                          |
| Sia   | 3.1              | ND              | 6.6                         |
| Fuc   | —                | ND              | 2.1                         |

*ND, not detected.*

### Notes

- Molar ratios are relative to Ser taken as 1.0.
- Molar ratios are relative to Thr taken as 1.0.
- ND, not done.
- Molar ratios are relative to Man taken as 3.0.
- Sia, sialic acid; Fuc, fucose.

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A variety of plant seeds and bacteria (4–6). The occurrence of endo-β-N-acetylgalcosaminidase in animal has been reported (16). However, the products formed by the action of endo-β-N-acetylgalcosaminidase do not retain a di-N-acetyllactosyl structure. Our previous findings of the accumulation at the late stage of oogenesis (1, 2) and the blastulation stage of embryogenesis (3)* of certain fish species of free N-glycan chains that retain a di-N-acetyllactosyl structure at their reducing termini strongly support the supposition that PNGase activity is expressed also in animal cells, and its expression may be significant for the metabolism and function of certain glycoproteins.

In other animal cells, however, the accumulation of glycoprotein-derived free oligosaccharides would not be observed since they are transported to lysosomes immediately after liberation from the parent glycoproteins and undergo further degradation catalyzed by glycohydrolases to their constituent monosaccharides (16). Our current interest is to find if PNGase activity appears stage-specifically during oogenesis and/or embryogenesis and to clarify the significance of de-N-glycosylation of phosvitin and hyosophorin in the fish embryos.
We have not systematically examined how the developmental expression of the presently found Medaka embryo’s PNGase changes during embryogenesis, differentiation, and ontogenesis. However, our preliminary results using 10 embryos in each experiment showed that during Medaka embryogenesis (from the 8-32-stage to gastrula via morula and blastula stages), the PNGase activity appeared to rise rather progressively to a maximum at the late blastula stage, followed a decay (we have not examined embryos later than gastrulation stage for PNGase activity). We detected PNGase activity even in unfertilized eggs or ovary, but no information is available if the PNGase activity present in the unfertilized eggs is originated from the PNGase identified and partially purified here. The cellular localization and developmental profile of the PNGase identified in this study would have to be substantiated once an antibody to this enzyme becomes available. Attempts to further purify the Medaka embryo PNGase are currently underway.

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