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KDN-GLYCOPROTEIN: A NOVEL DEAMINATED NEURAMINIC ACID-RICH GLYCOPROTEIN ISOLATED FROM VITELLINE ENVELOPE OF RAINBOW TROUT EGGS

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SUMMARY: A new acidic glycoprotein containing deaminated neuraminic acid (KDN = 3-deoxy-D-glycero-D-galacto-nonulosonic acid; >50%, w/w) was isolated from vitelline envelope of the unfertilized eggs of rainbow trout (Salmo gairdneri). This glycoprotein is designated as "KDN-glycoprotein" because it contains only KDN but no sialic acid as the acidic carbohydrate moieties. Other major carbohydrate components of KDN-glycoprotein were Gal and GalNAc. Thr and Ala accounted for 71% (mol/mol) of amino acid composition. A possible occurrence of KDN-KDN linkages, i.e. oligoKDN groups has been suggested in the carbohydrate chains presumably linked O-glycosidically to the core protein.

The only ulosonic acids found in glycoconjugates of animal source had been sialic acids until our recent discovery of KDN (Fig. 1A) in polysialoglycoproteins (PSGP) of rainbow trout eggs (1-3). For clarity, structures of three types of relevant ulosonic acids known to occur in nature are shown in Fig. 1. The 3-deoxy-D-manno-octulosonic acid (KDO; Fig. 1C) residues only occur in bacteria (4) and plant cell walls (5). Oligosaccharides containing KDN residues have been isolated from rainbow trout egg PSGP (2,3) and three types of KDN linkages, i.e. α-2$\rightarrow$8 to NeuGc residues, and α-2$\rightarrow$3 and α-2$\rightarrow$6 to GalNAc residues have been identified. KDN was shown to occur in every PSGP of salmonid fish eggs so far examined (3).

In this communication we report the isolation of another type of KDN-containing glycoprotein from vitelline envelope of rainbow trout eggs. This glycoprotein contains more than 50 % (w/w) KDN and no sialic acid so that it is unprecedented and we name it "KDN-

ABBREVIATIONS: KDN, 3-deoxy-D-glycero-D-galacto-nonulosonic acid; PSGP, polysialoglycoprotein; NeuGc, N-glycolyl-D-neuraminic acid; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid.

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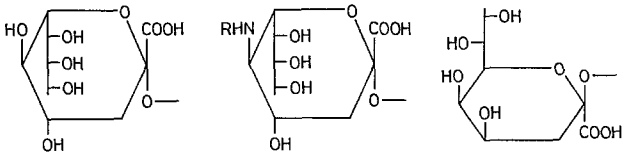

Fig. 1. Structures of (A) KDN (= 3-deoxy-D-glycero-D-galacto-nonulosonic acid), (B) sialic acids; NeuAc (R = $-COCH_3$) and NeuGc (R = $-COCH_2OH$), and (C) KDO (= 3-deoxy-D-manno-octulosonic acid).

glycoprotein". While KDN-glycoprotein is also a highly acidic glycoprotein, we find that it differs markedly from PSGP in terms of its carbohydrate and amino acid compositions and its subcellular localization.

EXPERIMENTAL Unless otherwise stated all isolation procedures were carried out in the cold. Unfertilized eggs (500 g) of rainbow trout (Salmo gairdneri) were crushed with a pestle and squeezed through gauze. Vitelline envelopes on gauze were washed free from bulk of yolk and cytoplasm by suspending in 0.8% NaCl (500 ml) and squeezing through gauze. The washed vitelline envelope was suspended in 4 volumes of 0.8% NaCl-2% SDS and the suspension was stirred on a magnetic stirrer for about 20 h at room temperature. To the filtrate through gauze was added 1 volume of 90% phenol and the mixture was stirred 4 h at room temperature. The aqueous phase obtained by centrifugation at 4,000 rpm for 20 min was dialyzed against distilled water in the cold and the dialyzed solution was lyophilized. The lyophilized material was further purified by gel chromatography on a Sephacryl S-200 (or S-300) column equilibrated and eluted with 0.1 M NaCl-0.1% SDS-0.01 M Tris-HCl (pH 8) and/or anion exchange chromatography on a DEAE-Sephadex A-25 column.

The presence of KDN-glycoprotein was monitored by the thiobarbituric acid (TBA) reaction (6) without prior hydrolysis. Neutral sugars were analyzed by the phenol-sulfuric acid method (7). Identification and quantitation of KDN and other sugars were carried out by gas chromatography (3). Amino acids and amino sugars were analyzed on a Hitachi KLA-5 amino acid analyzer after hydrolysis in vacuo in 5.7 N constant-boiling HCl at 110°C for 24 h (amino acids) or in 4 N HCl at 100°C for 4 h (amino sugars).

RESULTS AND DISCUSSION The presence of KDN-containing glycoprotein(s) in the vitelline envelope of the unfertilized eggs of rainbow trout was first evaluated by exhibiting typical color (λ_{max} = 549 nm) when a high molecular weight component was subjected to the thiobarbituric acid assay without prior hydrolysis. According to our previous experience with KDN-containing oligosaccharides obtained from PSGP, the bound KDN residues, unlike the bound sialic acid residues, are reactive in the thiobarbituric acid assay without hydrolysis. On the contrary, KDN gives no color in the resorcinol assay (8).

KDN-Glycoprotein was separated from other proteinaceous component, still remained after the phenol treatment of the vitelline envelope

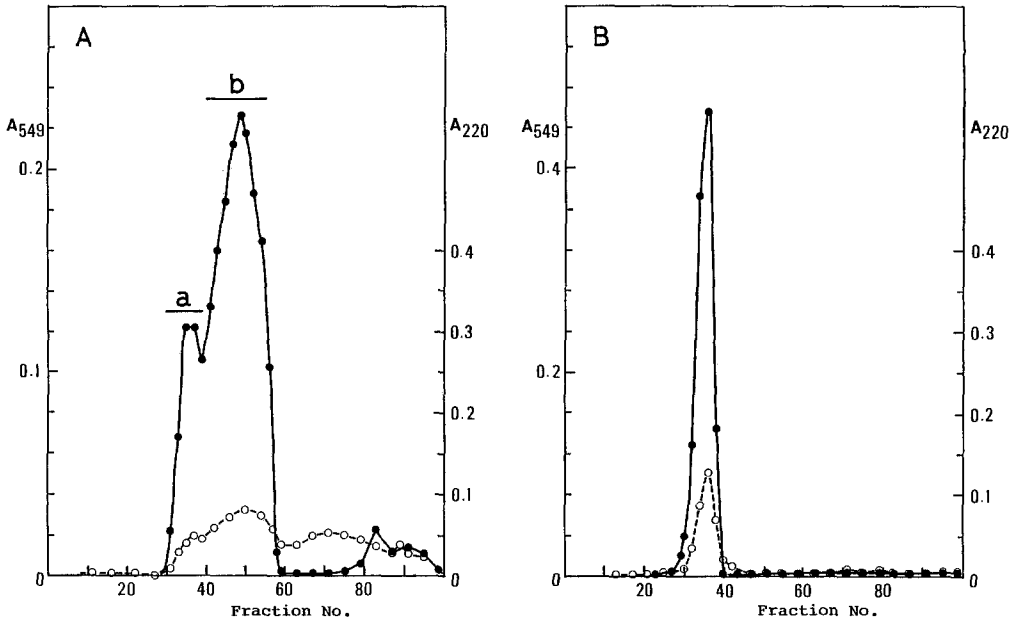

Fig. 2. (A) Sephacryl S-200 chromatography of KDN-glycoprotein in the extract of rainbow trout egg vitelline envelope and (B) rechromatography of the TBA-positive included material (**b**) on the same column. Sephacryl S-200 column (1.6x138 cm) was equlibrated and eluted with 0.1 M NaCl-0.1% SDS-0.01 M Tris-HCl (pH 8). Elution was monitored by absorbance at 220 nm (○) and the thiobarbituric acid assay (●).

extract, by Sephacryl S-200 chromatography (Fig. 2A). The TBA-positive peak eluted in the void volume (**a**) and slightly included (**b**) fractions contained KDN-glycoprotein. The included material was eluted as a sharp void volume-peak when rechromatographed on Sephacryl S-200 and also on Sephacryl S-300, indicating aggregative nature of KDN-glycoprotein (Fig. 2B), which may be involved in weak interactions with other component of the vitelline envelope. Further purification of KDN-glycoprotein was achieved by at least three rounds of repeated chromatography on Sephacryl S-200 (or S-300) until TBA-negative protein peaks eluted in retarded fractions no longer appeared. Only one KDN-glycoprotein peak, eluting at 0.4 - 0.5 M NaCl, was obtained by DEAE-Sephadex column chromatography. Although the anion-exchange chromatography was effective in purification of KDN-glycoprotein, the recovery was extremely low (<30%).

The purified KDN-glycoprotein migrated as a single diffuse band (apparent molecular weight, $>10^6$) on SDS/polyacrylamide gel electrophoresis when stained with either Stains-all or PAS. KDN-Glycoprotein was found not to be stained with Coomassie brilliant blue, and no other Coomassie blue-positive band appeared.

Table 1. Amino acid composition of KDN-glycoprotein from rainbow trout egg vitelline envelope

	mol/100 mol
Asx	3.0
Thr	43.3
Ser	6.5
Glx	1.7
Pro	4.1
Gly	2.7
Ala	27.6
Val	4.0
Met	0.2
Ile	1.6
Leu	1.4
Tyr	<0.2
Phe	<0.2
Lys	1.0
His	2.1
Arg	0.7

A typical preparation of KDN-glycoprotein contained about 81% carbohydrate (by weight %: KDN, >48; Gal, 14; Man, <2; GalNAc, 15; and GlcNAc, <2) and 19% protein, and was characterized by a large proportion of KDN (>48% by weight). The amino acid composition of the most purified sample is given in Table 1. Thr and Ala residues account for 71% of total amino acid residues. The carbohydrate composition suggests that O-linked carbohydrate units consisting of Gal, GalNAc, and KDN are predominant, although less frequent occurrence of N-linked carbohydrate unit(s) is also suggested by the presence of small amounts of Man and GlcNAc residues detected. Alkaline borohydride treatment followed by anion-exchange chromatography resulted in isolated of O-glycosidically linked KDN-containing oligosaccharides (data not shown). In view of aggregative nature of KDN-glycoprotein, the hydrophilic domain bearing these negatively charged carbohydrate units may possibly be separated from the naked peptide region responsible for intermolecular aggregation. It is noted that, in PSGP, the KDN residues occur as a minor component mostly at the non-reducing termini of poly(oligo)sialyl chains, whereas, in KDN-glycoprotein, no sialic acid was found and the observed molar ratio of KDN/(Gal + GalNAc) being 1.7 strongly suggests possible occurrence of KDN-KDN linkages. Despite the circumstantial evidence for the presence of oligoKDN groups the covalent nature of the linkages within KDN-glycan chains have yet to be identified.

The yield of purified KDN-glycoprotein was about 5 mg from 100 g eggs. The amount actually present in vitelline envelope would be at least doubled, i.e. at least 10 mg/100 g eggs, as judged by the TBA assay of the crude extract.

KDN was first discovered by us in PSGP of rainbow trout eggs. KDN-Glycoprotein was a new biomacromolecule quite different from PSGP both chemically and from subcellular localization: KDN was isolated from the washed vitelline envelope of unfertilized eggs, whereas PSGP has been established as a cortical vesicular component (9).

Although the biological function of KDN-glycoprotein is obscure, it may be that the KDN-glycoprotein has some, yet unknown, role in fertilization and the early embryogenesis, and may function as a barrier to bacterial invasion. Our interest in the KDN-glycoprotein stemmed from its unique structural features as well as its biological function. We have already succeeded in obtaining a sufficient amount for structural analyses of KDN-glycoprotein and raising antibody against it. Two interesting lines of future work on KDN-glycoprotein will probably be to study (a) its immunocytochemical localization, biological function and biosynthesis, and (b) its chemical structure.

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