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Identification of Hypocalcin (Teleocalcin) Isolated from Trout Stannius Corpuscles

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We have isolated and purified a glycoprotein from the corpuscles of Stannius (CS) of trout, which we consider hypocalcin (also called teleocalcin), the major hypocalcemic hormone of fish. This product is present in relatively large amounts in the CS of several species (i.e., European eel, tilapia, goldfish, and carp). Hypocalcin is typically released from the CS in response to an experimentally induced increase of the blood calcium concentration. Ultrastructural observations show that after this treatment the type I cells, reportedly the hypocalcin-producing cell type of the CS, are almost completely degranulated. The isolated glycoprotein has an apparent molecular weight of 54 kDa as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This molecule appears susceptible to breakdown and is recovered upon concanavalin-A affinity chromatography as a 41 kDa product. Reducing agents such as mercaptoethanol or dithiothreitol employed, e.g., during standard electrophoretic techniques or during amino acid sequence analysis, allow only the recovery of 28 or 18 kDa products. Evidence is given that the 54 and 41 kDa products are dimer molecules, with the 28 and 18 kDa products as their respective monomeric constituents. The sequence of the first 33 N-terminal amino acids of these products and the composition of the sugar component are presented. © 1988 Academic Press, Inc.

Corpuscles of Stannius (CS) are endocrine glands characteristic of holostean and teleostean fish. Since their discovery by Stannius (1839) a large body of histological research on the CS of many fish species has been carried out (Bauchot, 1953; Krishnamurthy, 1976; Krishnamurthy and Bern, 1969; Wendelaar Bonga and Pang, 1986). CS produce a factor that probably is the predominant hypocalcemic hormone in fish. Removal of the glands results in a strong increase in blood calcium concentration (e.g., Fontaine, 1964) that is reversed by reimplantations of CS or injections of CS tissue homogenates (Fontaine, 1967; Pang, 1973; Pang et al., 1973, 1974). Since the gills probably form the major site of calcium exchange between fish and water, inhibition of branchial calcium uptake may underlie hypocalcemic control (Fenwick, 1987; So and Fenwick, 1977, 1979).

There exists immunological resemblance between the hypocalcemic principle of CS and the parathyroid hormone (PTH) of the higher vertebrates (Lopez et al., 1981, 1984; Milet et al., 1982). Moreover, the CS hypocalcemic principle has similar bioactivity as PTH in mammalian and fish bioassays (Lafeber et al., 1986a, c; Milet et al., 1980; Verbost et al., 1986; Wendelaar Bonga et al., 1986). Although Wagner et al. (1986) reported on a hypocalcemic principle isolated from salmon, still much work has
to be done on the identity and mechanism of action of the hypocalcemic principle in fish.

CS of freshwater and euryhaline species contain two different cell types, type 1 and type 2 cells; the latter is absent in most seawater species (see review by Wendelaar Bonga and Pang, 1986). The fact that type 1 cells respond to changes in external calcium has led many researchers to postulate that these cells produce the hypocalcemic factor (Meats et al., 1978; Wendelaar Bonga et al., 1980; Wendelaar Bonga and Pang, 1986).

In 1976 Krishnamurthy suggested, on the basis of histological staining by periodic acid Schiff reagent, that the type 1 cells contain a glycoprotein. In 1978 Ma and Copp isolated a 3 kDa glycopeptide from salmon CS which they called teleocalcin; it was reported to be bioactive in vitro, by inhibition of branchial Ca\(^{2+}\)-stimulated ATP hydrolyzing activity, and in vivo, by its hypocalcemic action in eel (Copp et al., 1985). Pang and co-workers (1981), however, concluded from dialysis experiments that the bioactive factor of the CS of cod, which was called hypocalcin (Pang et al., 1986), was a product with a molecular weight of at least 13 kDa. Fenwick (1982) provided evidence that the hypocalcemic principle of eels had a molecular weight of at least 10 kDa. In our hands (Wendelaar Bonga et al., 1985) the hypocalcemic principle of tilapia appeared as a 28 kDa product when analyzed with electrophoretic techniques. Recently, Wagner et al. (1986) succeeded in purifying and characterizing a 39 kDa glycoprotein from salmon CS which showed inhibition of \(^{45}\text{Ca}\) uptake from the water in juvenile rainbow trout. The group of Milet et al. (1986) however, postulated the presence of two secretory substances in the CS. A 34 kDa peptide, which is called parathyrin of the corpuscles of Stannius (PCS), was partly purified and showed hypocalcemic bioactivity in stannectomized eels (Milet et al., 1986). A 70–80 kDa glycoprotein showing immunocross-reactivity with chromogranin A antisera and SP-1 (a secretory glycoprotein from the parathyroid cells) antisera was suggested to be co-secreted with PCS (Tissierand-Jochem et al., 1986; Lopez et al., 1986).

In this paper we report on the purification and partial identification of rainbow trout hypocalcin. It will be shown that trout hypocalcin is a 54 kDa glycoprotein, that is readily released upon increased plasma calcium levels.

MATERIALS AND METHODS

Stannius tissue homogenates. CS were obtained from rainbow trout (Salmo gairdneri), brown trout (Salmo trutta), European eel (Anguilla anguilla), tilapia (Oreochromis mossambicus), goldfish (Carassius auratus), and carp (Cyprinus carpio). The glands were homogenized in 0.05 M ammonium acetate (pH 7.4) using a Potter homogenizer fitted with a Teflon pestle. The supernatant obtained after centrifugation (5 min; 9000g) was lyophilized and prepared for sodium decyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see below).

Calcium injection. Rainbow trout and European eel were injected intraperitoneally with 0.68 M CaCl\(_2\) solution (100 \(\mu\)l/100 g fish/day) for 2 days. Injections of NaCl solutions of identical molarity served as controls. Four hours after the last injection blood was collected by puncture of the blood vessels of the caudal peduncle using a heparinized syringe (Ca\(^{2+}\)-heparin, Radiometer). Subsequently the CS were removed and prepared for SDS–PAGE. Blood was analyzed for ionic calcium and pH; plasma was analyzed for total calcium and osmolality. From every fish, part of the CS tissue was fixed and stained for electron microscopy as described by Wendelaar Bonga et al. (1980). For the discrimination between type 1 and type 2 cells structural parameters as described by Wendelaar Bonga et al. (1980) were used.

Analytical methods. Plasma total calcium and phosphate were determined with a commercial calcium kit (Sigma). Combined calcium/phosphate standards (Sigma) were used as a reference. Protein content was estimated with a commercial reagent kit (Bio-Rad) using bovine serum albumin (Bio-Rad) as a reference. Blood ionic calcium concentration and blood pH were determined using an automated ionic calcium analyzer (Radiometer). Osmolality was measured using a Roebling microosmometer. Distilled water and an osmolality standard of 300 mOsmol/kg (Sigma) were used as standards.
SDS–polyacrylamide gel electrophoresis. SDS–PAGE was performed according to Laemmli (1970) with 15% polyacrylamide slab gels. To investigate the effects of reducing agents on the CS principle, SDS–PAGE was carried out under reducing conditions (with mercaptoethanol) or nonreducing conditions (without mercaptoethanol). After fixation of the proteins in the gels by methanol and glutaraldehyde, the gels were silver-stained (Morrissey, 1981). Data were quantified by densitometer scanning using a Bio-Rad Model 1650 transmittance scanning densitometer.

Isolation procedure. The isolation of the hypocalcemic principle from CS homogenates was started with concanavalin A–Sepharose 4B column chromatography (Sigma; 1.77 cm² × 5 cm). The column was equilibrated with 0.015 M Tris buffer, pH 7.4, containing 1 mM each MnCl₂, MgCl₂, and CaCl₂, and 1 M NaCl (Con A buffer) according to Roezlzema and Van Erp (1983): the flow was 15 ml/hr at 4°C. Lyophilized whole tissue homogenates of rainbow trout were kept in stock at 20°C. Approximately 100 mg dry wt tissue (equivalent to 400 mg wet wt obtained from 40 kg trout) was homogenized in 3 ml Con A buffer with a Potter-type homogenizer fitted with a Teflon pestle. The supernatant obtained after centrifugation (5 min; 9000 g) was applied to the column and passed three times through the column by short-circuiting the system. Products not bound to Con A (residue) were eluted with Con A buffer. The product binding to Con A were eluted with 0.3 M α-methyl-D-glucoside in Con A buffer (isolated product).

Desalting and concentration of eluted material were carried out by ultrafiltration (Amicon Inc). Diaflow YM-10 membranes were used for the Con A binding material and YM-5 membranes for the residue. Ultrafiltration was carried out at 4°C in 180-ml stirred cells operated at 2.6 MPa N₂. After the volume of the eluates had decreased to about 10 ml, the ultracentrifugation cells were refilled with ammonium acetate buffer (0.05 M; pH 7.4) to 180 ml; this procedure was repeated three times. By so doing the Con A buffer was diluted over a thousand times. The remaining 10 ml was lyophilized in fractions as required for assays and subsequently stored at −20°C. SDS–PAGE of the samples was routinely carried out as a quality check for product composition. The samples containing the presumed hypocalcemic principle proved to be pure for at least 95% on protein basis.

HPLC analysis of the Con A binding fraction was carried out using a ChromSpher C-18 reversed-phase HPLC column (Chrompack). Elution was performed with an acetonitrile gradient (0–50%) in a buffer containing sodium–potassium tartrate (5 mM; pH 3.0), Na₂SO₄ (50 mM), and butanesulfonic acid (5 mM); the flow was 1 ml/min. Eluted material was detected with a Spectraflow 783 absorbance detector at 280 nm. The fraction containing a significant amount of protein was collected. Freeze drying and trichloroacetic acid precipitation methods were used to remove acetonitrile and salts from this sample before SDS–PAGE was performed.

Amino acid sequence analysis. Amino acid sequences were determined at the Gas Phase Sequencer Facility (Department of Medical Biochemistry, State University of Leiden, The Netherlands). The instrument used was an Applied Biosystems Model 470A protein sequencer, on-line equipped with a Model 120A PTH analyzer. Pyridyl-ethylation (Friedman et al., 1980) was carried out for cysteine identification. Amino acid sequence analysis was carried out in the presence of the reducing agent di-thiothreitol.

Carbohydrate analysis. Carbohydrate analysis of the isolated protein was performed at the University of Alberta, Department of Physiology, according to Dutton et al. (1978). Data were quantified by gas–liquid chromatography–mass spectrometry, using xylose as an internal standard.

Statistical analysis. Data are presented as mean values ± SD. Statistical evaluation was performed by the use of the unpaired Student's t test. Significance was accepted at P < 0.05.

RESULTS

Identification of the hypocalcemic factor. Figures 1 and 2 show typical examples of densitometric scans of silver-stained products, after SDS–PAGE under reducing conditions, present in a crude tissue homogenate of CS of six different teleost fish. A product with an apparent molecular weight of approximately 28 kDa was consistently observed. In Fig. 2 it is shown for trout and eel that the 28 kDa protein disappeared almost completely from CS tissue homogenates after CaCl₂ injection. Typically, the 28 kDa protein constitutes 20% of the total amount of protein present in the CS tissue homogenate of these fish, as derived from analysis of the area under the curve of the densitometric scans of the silver-stained SDS gels. A densitometric scan after SDS–PAGE of a crude tissue homogenate of NaCl-injected fish did not differ from a control CS tissue homogenate.

Electron micrographs (Figs. 3 and 4) show that the CS of the untreated and the NaCl-injected fish contain an abundance of
large secretory granules. These granules are typical for the type 1 cells (Wendelaar Bonga et al., 1976; Meats et al., 1978). The cells of the CS of the CaCl₂-injected fish are almost devoid of these secretory granules.

Total plasma calcium levels increased significantly in the CaCl₂-injected trout and eel (2.47 ± 1.12 to 3.10 ± 0.30 and 3.08 ± 0.16 to 6.14 ± 0.41 mM, respectively), but were unchanged in the NaCl-injected controls. Plasma osmolality and blood pH did not change significantly in either group.

Figure 5 shows a crude tissue homogenate of trout after SDS-PAGE under reducing and nonreducing conditions. Figure 5 (top) shows that this product has an apparent molecular weight of 28 kDa only after electrophoresis under reducing conditions. Surprisingly, under nonreducing conditions this product showed an apparent molecular weight of approximately 54 kDa (Fig. 5 bottom).

Isolation. Figure 6 shows a densitometric scan after SDS-PAGE of a crude CS tissue homogenate and the fractions after isolation. As shown the major constituent of the Con A binding material has an apparent molecular weight of approximately 18 kDa under reducing conditions (Fig. 6 bottom). A minor contamination (with a molecular weight of approximately 55 kDa) is observed in this fraction; this contamination is present in considerable amounts in

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**Fig. 1.** Densitometric scan of silver-stained CS crude homogenates after SDS-PAGE of goldfish, carp, tilapia, and brook trout. Arrow indicates 28 ± 2 kDa peak for all species. Absorbance is shown as percentage of maximum peak height (= 100%) and corresponds to the amount of material. Molecular weights (m) of protein markers are given on the horizontal axis.

**Fig. 2.** Densitometric scan of silver-stained CS crude homogenates of NaCl- (C) or CaCl₂- (E) injected eel and trout after SDS-PAGE. Absorbance is shown as percentage of maximum peak height (= 100%). Molecular weights (m) of protein markers are given on the horizontal axis.
**Fig. 3.** Electron micrographs of CS of NaCl- (C) or CaCl₂- (E) injected trout; C, type 1 cells, with many large secretory granules; E, type 1 cells devoid of secretory granules (15,000×).
Fig. 4. Electron micrographs of CS of NaCl- (C) or CaCl₂- (E) injected eel; C, type 1 cells, with many large secretory granules; E, type 1 cells devoid of secretory granules (15,000×).
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the residue. The residue, however, does not contain the product present in the isolated fraction (18 kDa), or the products that are released from the CS after calcium injections (28 kDa). Under nonreducing conditions (Fig. 6 top) the Con A binding material has a molecular weight of approximately 41 kDa.

Protein analysis of the isolated fractions showed that from a tissue homogenate of 100 mg dry wt of CS (with a protein content of 25.3 ± 3.6 mg) 3.07 ± 0.56 mg (12%) of isolated product and 6.19 ± 0.84 mg (24%) of residue protein was recovered (n = 15).

Reversed-phase HPLC analyses performed to purify further the Con A binding material yielded a single symmetrical peak at 50% acetonitrile, indicating high polarity of the product. A minor peak eluted at 35% acetonitrile, representing less than 5% of the total amount of protein. The 50% acetonitrile peak has an apparent molecular weight of 54 kDa (Fig. 7).

Amino acid analysis and carbohydrate analysis. Figure 8 shows the sequence of the first 33 N-terminal amino acids of rainbow trout isolated Con A binding product. No differences in the N-terminal amino acid sequence were observed between the 28 and the 18 kDa product. At position 7 serine and glutamic acid were detected consistently indicating a microheterogeneity in these products. Position 29 could not be identified due to a high polarity of this site. This indicates the presence of a sugar residue linked to the amino acid chain. Carbohydrate analysis revealed that the
sugar residue consisted of mannose, galactose, and glucosamine, in the ratio of 1:1:2. The total sugar content made up 12% of the 54 kDa protein on a weight basis.

**DISCUSSION**

In this study we show the isolation of a glycoprotein from the CS of trout. This product is the hypocalcemic factor of the CS since the release of this glycoprotein from the CS is stimulated substantially and specifically upon experimentally induced increase of the blood calcium levels. Furthermore it is shown that this product inhibits branchial calcium influx in trout, which has been suggested to be the main effect of the CS hypocalcemic hormone (Lafeber et al., 1986b). Because of its high molecular weight we will further call this hypocalcemic glycoprotein from the CS of trout "hypocalcin." This name was proposed for a CS hypocalcemic principle with a molecular weight above 10 kDa by Pang et al. (1974, 1981), whereas the name teleocalcin was first given to a hypocalcemic principle with a molecular weight of 3 kDa isolated from the CS of salmon (Ma and Copp, 1978).

Hypocalcin is present in relatively large amounts in the CS of the six freshwater species investigated. We conclude that hypocalcin, which appears as a 54 kDa band upon SDS–PAGE, is composed of two similar subunits that appear as a 28 kDa band upon SDS–PAGE under reducing conditions. We further conclude that during isolation using Con A chromatography the C-terminal part is split off of the two subunits resulting in the appearance of an 18 kDa band upon SDS–PAGE under reducing conditions. After SDS–PAGE under nonreducing conditions this product appears as a dimer identified as a 41 kDa band. The following arguments have led us to these conclusions.

Corpuscles of Stannius of most freshwater teleost contain two different cell types: type 1 cells, which predominate in number and contain the large secretory granules, and type 2 cells, which contain the small secretory granules (see review by Wendelaar Bonga and Pang, 1986). In the literature, type 1 cells are presumed to contain
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the hypocalcemic principle, since only type 1 cells respond to changes in external calcium concentration (Wendelaar Bonga et al., 1976, 1980; Meats et al., 1978). In the present study we showed that an experimentally induced increase in plasma calcium was accompanied by massive release of the granules of the type 1 cells, as well as by the almost complete disappearance of the 28 kDa product from the CS. These observations lead to the conclusion that the isolated product represents the hypocalcemic principle of the CS.

The present observation on the molecular weight of 28 kDa for the hypocalcemic principle of trout and eel are in agreement with previous reports from our laboratory on the molecular weight of the CS hypocalcemic principle of the tilapia O. mossambicus (Wendelaar Bonga et al., 1985). A similar 28 kDa product is present in relatively large amounts in the CS of all species investigated in the present study. This 28 kDa product is synthesized and released during in vitro incubations of tilapia CS, as was shown by radioactive amino acid incorporation experiments (Wendelaar Bonga et al., 1985). Nevertheless we propose that the hypocalcemic principle of CS is a 54 kDa glycoprotein. An apparent molecular weight of 28 kDa is, however, observed upon SDS-PAGE under reducing conditions.

How does hypocalcin with an apparent molecular weight of 28 kDa, obtained by SDS-PAGE under reducing conditions, relate to the 54 kDa protein, obtained by SDS-PAGE under nonreducing conditions? We suggest that hypocalcin is composed of two subunits of similar molecular weight which dissociate under reducing conditions. The following results support this hypothesis. Under reducing conditions the 54 kDa hypocalcin appears as a single band of 28 kDa. Furthermore, the results of the amino acid sequence analysis also point to the presence of almost identical subunits. This amino acid analysis is performed under reducing conditions. This implies that amino acid sequence analysis of the 54 kDa hypocalcin in fact concerns analysis of the 28 kDa subunits. Only a single N-terminal sequence is obtained. An identical sequence is obtained after analysis of the isolated 41 kDa hypocalcin, which actually represents the N-terminal sequence of the 18 kDa subunits obtained under reducing conditions. However, a consistent microheterogeneity was observed at position 7 of native 54 kDa as well as the isolation variant, the 41 kDa product. We suggest therefore that trout hypocalcin consists of two similar subunits that only differ at position 7. The high incidence of cysteines (4 in the N-terminal 33 amino acids) makes the presence of disulfide bridges between the two subunits in the native molecule likely. From their recent work on the salmon hypocalcemic principle, Wagner and colleagues (1986) also concluded that the salmon CS hypocalcemic principle could be composed of two subunits.

Isolation of hypocalcin yields a band of 41 kDa after SDS-PAGE under nonreducing conditions, and of 18 kDa under reducing conditions. These purified 41 and 18 kDa products are obviously related to the 54 and 28 kDa products observed in freshly prepared homogenates of CS tissue. We suggest that the 18 kDa product is the N-terminal part of the 28 kDa product. The following results support this hypothesis. A tissue homogenate containing the 28 kDa hypocalcin yields an 18 kDa product upon isolation. The residue is consistently devoid of both the 28 kDa and the 18 kDa product. Furthermore, for still unknown reasons in about 1 out of 10 isolations only a 28 kDa product is isolated instead of an 18 kDa product and under nonreducing conditions only a 54 kDa product is obtained instead of a 41 kDa product. Prolonged storage of lyophilized CS tissue does result in the disappearance of the 28 kDa hypocalcin and the concomitant appearance of
an 18 kDa product. Although our isolation is carried out at 0° we assume that endogenous protease activity converts hypocalcin from the 28 kDa to the 18 kDa product. Perhaps the most convincing evidence for the suggested relationship between the 28 and 18 kDa products is the fact that amino acid sequence analysis of both purified products reveals identical results for the first 33 N-terminal amino acids determined so far (see below). Therefore the 18 kDa product must be a N-terminal portion of the 28 kDa molecule which is split off during the isolation procedure.

Hypocalcin in the isolated fraction, obtained by Con A chromatography is slightly contaminated, as is shown by SDS-PAGE as well as by HPLC analysis. On SDS gel these contaminants (less than a few percent on protein basis) are abundantly present in the residue. The homogeneous "50% acetonitrile peak" collected by reversed-phase HPLC analysis appears to be hypocalcin and to be free of contaminants.

The molecular weight of 41 kDa of the trout isolated product is very close to the molecular weight of 39.3 kDa reported for the presumptive hypocalcemic factor from the CS of salmon (Wagner et al., 1986). We show here, however, that the molecular weight of the 41 kDa product isolated from trout is an artifact and that the native product is a molecule of 54 kDa. It is possible that the molecular weight of 39.3 kDa reported for the hypocalcemic principle of salmon also applies to the N-terminal part of a larger molecule.

The first 33 amino acids of trout hypocalcin show remarkable overlap with the first 19 amino acids of the isolated glycoprotein from salmon reported by Wagner et al. (1986) and the amino acid sequence of the CS principle of eel (A. Butkus, personal communication). Differences with salmon were found only on positions 3 and 18 where serine and alanine are substituted for proline and aspartic acid, respectively. For eel, differences were found at the same and at five additional positions (position, trout/eel; 3, Ser/Ala; 4, Asn/Ser; 18, Ala/Glu; 21, Gly/Ser; 22, Thr/Ala; 28, Gln/Asp; 33, Asp/Asn). All substitutions could be due to single mutations, except for Ala and Gln, position 18 for trout and eel, respectively. For salmon the amino acid on position 12 has not been identified. For trout hypocalcin, a cysteine is found on position 12. For salmon no cysteines were detected (Wagner et al., 1986). Therefore, it may well be that salmon hypocalcemic principle has a cysteine on position 12, and that differences in the first 19 amino acids exist only at positions 3 and 18.

The amino acid on position 29 could not be determined for our isolated trout hypocalcin due to the high polarity of this position. This indicates the presence of a carbohydrate chain. Support for this hypothesis comes from the finding that position 31 is a threonine, since linkage of N-acetylglucosamine to aspartic acid (the most common binding) requires a sequence Asn-Xxx-Thr/Ser (Bahl and Shah, 1977). Consequently, this would result in an Asn on position 29. For eel, on this position an Asn is also found (A. Butkus, personal communication). Presently, however, we can not exclude the possibility of an oligosaccharide O-linked to this position, which indicates the presence of a serine or threonine. The ratio of mannose, galactose, and glucosamine (1:1:2) that we found for trout deviates from the carbohydrate composition of the hypocalcemic principle of salmon (Wagner et al., 1986). We found a higher content of galactose and did not detect any glucose.

Lopez, Milet, and co-workers (Lopez et al., 1986; Milet et al., 1986; Tisserand-Jochem et al., 1986) suggested that in the CS of eels of glycoprotein is cosecreted with a proteinaceous hypocalcemic principle, and that this glycoprotein is similar to secretory protein-1 (SP-1) from the parathyroids of terrestrial vertebrates. We showed however that the isolated glycoprotein of trout is hypocalcemic and possesses
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PTH-like bioactivity (Lafeber et al., 1986a, b). In this respect our results on trout are in line with those of Wagner et al. (1986) on salmon, who also concluded the hypocalcemic product is a glycoprotein.

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