Identification and Study of Gangliosides from *Scomber scombrus* Muscle

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Scomber scombrus has been implicated in the disease known as scombroid food poisoning (histamine intoxication). However, some investigators claim that scombroid food poisoning is not caused by histamine only. Gangliosides have been found in different type fishes, but in all cases the sources are brain, melanoma, retina, and optic nerve. These complex lipids as well as their derivatives, exhibit important biological activities. In this study, gangliosides (8×10^{-4} % w/w in muscle) were isolated from *S. scombrus* muscle for the first time, and the major one was found to be monosialoganglioside. Gangliosides existed as a proteolipid type complex combined with a protein consisting of high amounts of iron and copper and which seemed to be the red protein. After fractionation of gangliosides onto cation exchange, silica, and C_{18} HPLC columns, we detected two compounds which induced aggregation through platelet-activating factor (PAF) way. Both of them were eluted onto HPLC in the region of gangliosides and from the results of the biological experiments as well as from chemical determinations, they seemed to be *O*-acetyl derivatives of gangliosides. These molecules could partly contribute to scombroid food poisoning since the main symptoms of this disease are well-known actions of PAF.

Keywords: *Gangliosides; Scomber scombrus; platelet-activating factor, PAF; biological activity; fish muscle*

INTRODUCTION

Scomber scombrus has been implicated in the disease known as scombroid food poisoning (histamine intoxication), hypotension and hemocondensation being the main symptoms. Until now the above disease was attributed to the existence of high levels of histamine—a well-known mediator of allergy and inflammation—in this fish which were derived from histidine decarboxylation by the fish microbial greenfinch (*Proteus, Klebsiella*, etc.) (Ferencik, 1970). However, some investigators claim that scombroid food poisoning should not be caused by histamine only. On the other hand, platelet-activating factor, PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a biologically active ether phospholipid, is biosynthesized by a variety of animal cell types and exhibits a wide spectrum of biological actions being the most potent platelet aggregating factor known today (Lee and Snyder, 1985). Among the *in vivo* biological actions of PAF are histamine secretion, hypotension, and hemocondensation (Lee and Snyder, 1985), which the main symptoms of the scombroid food poisoning. Lipids with PAF-like activity *in vitro*, present in *S. scombrus*, may act *in vivo* either directly to the cells or/and with a secondary action, through the secretion of other inflammatory mediators (e.g., histamine). The existence of such molecules in this fish, could partly explain the symptoms observed in scombroid food poisoning.

Gangliosides are the most complex glycolipids. These molecules are found in the outer half of the membrane bilayer and their sugar groups are exposed at the cell surface, suggesting some role in the interactions of the cell with its surroundings. Gangliosides are most abundant in the plasma membrane of nerve cells, where they constitute 5−10% of the total lipids, although they are found in much smaller quantities in most cell types with the exception of tumour cells, where they exist in higher amounts. In most cases, gangliosides occur in free form but there is also evidence indicating their occurrence in complex forms with proteins. Milk gangliosides are reported to occur as a proteolipid type complex where the protein residue is the so-called red protein of milk (Kapoulas et al., 1975).

Gangliosides have been found in different type fishes, but in all cases the sources are brain, melanoma, retina, and optic nerve (Tanaka et al., 1989; Felding-Habermann et al., 1988; Sonnentag et al., 1992; Rahmann et al., 1992). These complex lipids as well as their derivatives, either naturally occurring ones (such as *O*-acylated) or breakdown products after chemical treatments, exhibit important biological activities (Felding-Habermann et al., 1988; Nalivaeva et al., 1987). It is reported that some gangliosides stimulate a variety of inflammatory cells such as neutrophils and platelets, others show platelet anti-aggregating properties *in vivo* and *in vitro* and also inhibit platelet adhesion (Ferroni et al., 1995; Tubaro et al., 1994; Valentino and Ladisch, 1994; Tubaro et al., 1993). Gangliosides may also be involved in the inflammatory reaction which induces the atheromatosis generation since they are accumulated selectively in the intima of atherosclerotic vessels (Mazurov et al., 1988). It has also been described the acetylation/deacetylation mechanism for a monosialoganglioside which is involved in the epidermal growth factor activity regulation (Hanai et al., 1988).

The above observations are in accordance with our findings which demonstrate that acetylation of gangliosides resulted in compounds with a variety of biological activities *in vitro* and *in vivo*, similar to the ones of PAF (Tournis et al., 1986).

In this study, we isolated gangliosides from *S. scombrus* muscles and studied their biological activity in washed rabbit platelets.

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MATERIALS AND METHODS

Solvents and Reagents. All reagents and chemicals were of analytical grade supplied by Merck (Darmstadt, Germany). High-performance liquid chromatography (HPLC) solvents were purchased from BDH (Poole, U.K.) and LABSCAN (Doublin, Ireland). Lipid standards were obtained from Supelco (Bellefonte, PA). Chromatographic material used for thin-layer chromatography was silica gel G-60 (Merck, Darmstadt, Germany). Semisynthetic PAF (80% C-16PAF and 20% C-18PAF) was synthesized in our laboratory as previously described (Demopoulos et al., 1979). Bovine serum albumin (BSA) and thrombin were purchased from Sigma (St. Louis, MO). Human plasma acetylhydrolase was a gift from Dr. D. Stafforini.

Instrumentation. HPLC was performed on a Varian Star model, series 9000, (Walnut Creck, CA), supplied with a 250 μL loop Rheodyne (P/N 7125) injector. A Varian model UV-vis/9050 spectrophotometer was used as detector. The spectrophotometer is connected to a Metter GA17 plotter (Gravency, Zurich, Switzerland). The following columns were used: a cation exchange column SS 10 μm Partisil 25 cm $\times$ 4.6 mm i.d., PXS 10/25 SCX from Whatman (Clifton, NJ) with a gradient elution system that consisted of A, methanol/water (4:1, v/v), 25%, and B, acetonitrile, 75%, for 10 min, followed by a linear gradient to A:B 50%:50% for 40 min. The flow rate was 1.5 mL/min, and the UV-detector was at 210 nm. A reverse phase column 10 μm Bondapack C18 25 cm $\times$ 4.6 mm i.d., from Waters (Milford, MA) with a gradient elution system consisted of a linear gradient from methanol/water, 4:1 (v/v), to acetonitrile/ methanol, 7:5 (v/v), in 10 min and hold in the last solvent mixture. The flow rate was 1 mL/min and the UV detector was at 205 nm. An absorption column, Silica 25 cm $\times$ 4.6 mm i.d., from Hichrom H5 (Reading, Berkshire, England) with a gradient elution system that consisted of acetonitrile 100% for 10 min, followed by a linear gradient to methanol 100% for 20 min, and finally a hold in methanol for 40 min was used. The flow rate was 2 mL/min, and the UV detector was at 254 nm.

The PAF-induced aggregation was measured in a Chrono-Log (Havertown, PA) aggregometer coupled to an Omniscribe recorder (Houston, TX).

UV spectra were recorded on a UV−vis, Uvicon 942 spectrophotometer (Kontron, Milan, Italy) with a Citizen recorder, model Swift 90 (Middle Essex, U.K.). The spectrum (190−800 nm) along with the second to fourth derivatives, was recorded with 1 cm path length against a blank using a double-beam recording spectrophotometer.

Atomic absorption spectra were recorded on a A30 Varian (Walnut Creek, CA). The sample was digested with a mixture of HNO_3 and HCl (2:1, v/v) and heated for 15 min at 90 °C. The temperature was increased at 170 °C and the sample remained for 5 min at this temperature. After cooling, the iron and copper were measured in a clear solution with the help of a calibration curve.

Sample Preparation. The samples (*S. scombrus*) were fished from Greek seas and identified at the Institute of Fish Technology, Agriculture Ministry, by M. Alexadropoulos. Muscle was obtained from quantity of 10 kg of fish (average weight per fish, 300 g) and kept at 0 °C. After homogenation with Ultra-turrax, model IKA TP 18/10 (Staufen, Germany), for 10 min, the sample was divided into two parts. The first one was used in order to estimate the sample freshness by measuring total volatile nitrogen by the method of Kirk and Sawyer (1991), thiobarbituric acid by the method of Kirk and Sawyer (1991), and histamine (AOAC, 1990). The other one was used for obtaining total lipids by the method of Bligh-Dyer (1959).

Chemical Determinations. Hexose expressed as glucose was determined by the method of Galanos et al. (1965). *N*-Acetylneuraminic acid (sialic acid) determination was performed by the method of Svennerholm (1957). Ester determination was performed by the method of Snyder and Stephens (1959).

Biological Assays. The PAF-like activity of the examined samples was estimated by bioassay studies based on washed rabbit platelets (Demopoulos et al., 1979). PAF and the examined samples were dissolved in 2.5 mg of BSA per mL of saline. Thrombin was dissolved in saline. Various concentrations of the examined samples were added into the aggregometer cuvette and the aggregation induced by the samples was studied in a Chronolog aggregometer. Experiments with specific inhibitors, creatine phosphate (CP) 0.7 mM/creatine phosphate kinase (CPK) 13 units per mL of saline, indomethacin 10 μM (10% ethanol in water), and BN52021 0.1 mM (0.3% dimethyl sulfoxide, DMSO, in water), were also performed. These inhibitors were added to washed rabbit platelets 1 min prior to the addition of the examined sample into the aggregometer cuvette. This experiment was carried out according to Lazanas et al. (1988).

The platelet aggregation induced by PAF (1.25×10^{-10} M, final concentration) was measured as PAF-induced aggregation, in washed rabbit platelets before and after the addition of various concentrations of the examined sample. This experiment was also performed with thrombin (0.125 units/cuvette) in order to assess the inhibition of thrombin-induced aggregation.

Desensitization Experiment. This experiment was carried out according to Lazanas et al. (1988). Briefly, in desensitization and cross-desensitization experiments, platelets were desensitized by the addition of PAF or the examined sample to the platelets suspension at a concentration which caused reversible aggregation. Second stimulation was performed immediately after complete disaggregation with the addition of the examined lipid or PAF or thrombin, all added at concentrations which induced platelet aggregation pattern of the same height with the first one.

Treatment with Acetylhydrolase. In a test tube prewarmed at 37 °C phosphate buffer (0.08 M, pH 7.5) was added along with human serum acetylhydrolase, purified by Dr. D. Stafforini (Stafforini et al., 1987), and the sample was in 2.5 examined mg of BSA per mL of saline. The enzymatic system was incubated at 37 °C, and at different times aliquots were taken and tested for their ability to induce washed rabbit platelet aggregation.

RESULTS AND DISCUSSION

The experimental procedure of the isolation and purification of gangliosides from *S. scombrus* fish muscle, is shown in Figure 1.

An amount of total lipids was separated into neutral lipids and polar lipids by current counter distribution (Galanos et al., 1962). Polar lipids were fractionated onto HPLC cation exchange column with the elution system described in materials and methods.

The area between 20 and 40 min of the above fractionation (fraction X) was collected, and during evaporation under a stream of nitrogen, a blue-white precipitate appeared while the supernatant became green. These observations indicated the presence of ganglioside−protein complex. The residue was redissolved in chloroform/methanol, 1:1 (v/v), and the precipitate was removed by centrifugation at 1000*g*. In the precipitate, protein was detected (47 μg/mL) by the method of Lowry et al. (1951). This protein had also the capacity of binding iron from alkaline solutions and obtaining a salmon red color.

The supernatant obtained after the above centrifugation, was chromatographed along with standard gangliosides, on TLC plates using propyl alcohol/water, 7:3 (v/v) as developing system, and the plate was sprayed with resorcinol reagent (Figure 2). The sample was mainly consisted of monosialogangliosides (G_4) and of minor amounts of disialogangliosides (G_3, G_2) with R_f values of 0.40, 0.30, and 0.22, respectively. A red band also appeared with R_f 0.19, which should be attributed to the presence of the well-known red protein (Groves, 1971).

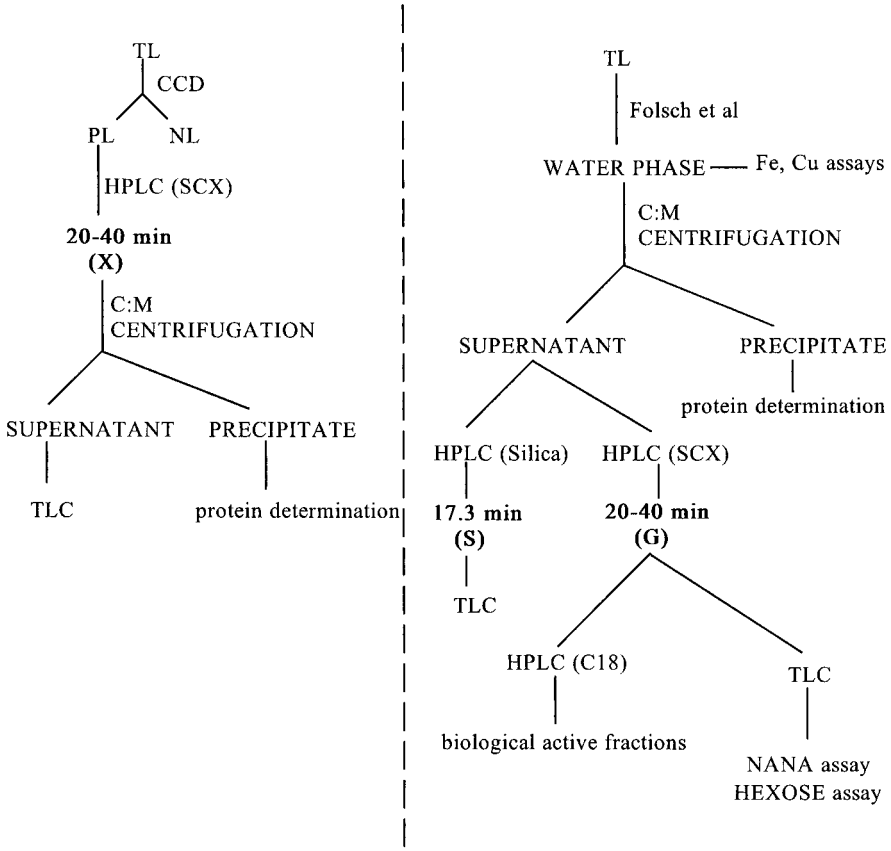

Figure 1. Diagram of the experimental procedure of the isolation and purification of gangliosides from *S. scombrus* fish muscle. TL, total lipids; PL, polar lipids; NL, neutral lipids; CCD, current counter distribution; C, chloroform; M, methanol; X, S, and G, ganglioside fractions cited in Results and Discussion.

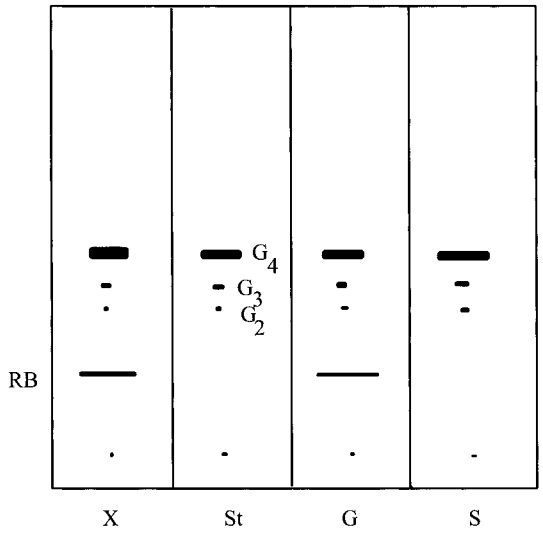

Figure 2. TLC of ganglioside fractions X, S, and G along with standard gangliosides. G_4, monosialo-gangliosides; G_3, G_2, disialogangliosides; RB, red band corresponding to the red protein. Developing system, propyl alcohol/water, 7:3 (v/v). Visualization with resorcinol reagent.

The chromatographic behavior of the fraction X on cation exchange HPLC column and on TLC, along with the protein determination, led to the assumption that a proteolipid type complex should be present in this fraction. The lipid part of the above complex seemed to be gangliosides while the protein part should be the red protein. This proteolipid type complex is separated into protein and lipids during evaporation where the proteolipid bonds are broken.

In order to confirm the above findings, we isolated gangliosides (about 700 μg) from total lipids (10 g) by the method of Folch et al. (1957). The water phase which contained gangliosides was evaporated to dryness under a stream of nitrogen and redissolved in chloroform/methanol, 2:1 (v/v). The precipitate with blue-white color was removed by centrifugation. In the precipitate, we determined protein which was approximately 20% of the total quantity of this fraction (138 μg of protein out of 700 μg of the fraction).

The major quantity of the supernatant was fractionated onto cation exchange HPLC column with the conditions used in the fractionation of polar lipids. The area between 20 and 40 min was collected and will be referred as fraction G. Fractions G and X along with standard gangliosides were chromatographed on silica plates using the same development system as above (Figure 2). Both fractions had identical chromatographic behavior on TLC and on HPLC, where only one peak appeared at approximately 29.5 min which revealed the presence of gangliosides as well as the existence of the red protein.

A small quantity of the supernatant was fractionated onto silica HPLC column with the elution system described in materials and methods. A major peak (fraction S) eluted at 17.3 min sharing the same retention time as standard gangliosides which were eluted from 17−17.8 min. This peak (S) was chromatographed on TLC along with standard gangliosides and fraction G with the development system mentioned above (Figure 2). The examined peak consisted mainly of monosialogangliosides (G_4) and of minor amounts of disialogangliosides (G_3, G_2) with R_f values of 0.40, 0.30, and 0.22, respectively. As expected, there was total absence

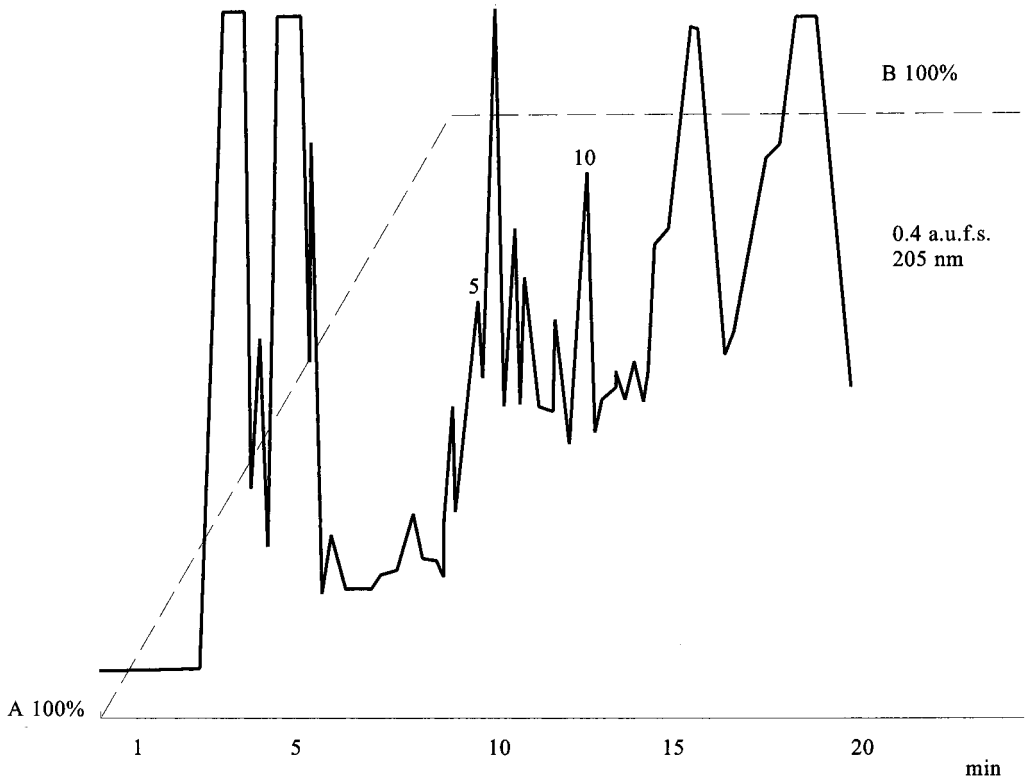

Figure 3. HPLC fractionation of gangliosides from *S. scombrus* using a Bondapack C_{18} column. A, methanol/water, 4:1 (v/v); B, acetonitrile/methanol, 7:5 (v/v). The peak numbers 5 and 10 refer to the two fractions which induce washed platelet aggregation.

of red band on the above TLC of the examined peak since it is known that proteolipid bonds are broken in the presence of polar compounds such as the silica HPLC column.

In order to confirm the above findings and to determine the molar ratio of hexose to *N*-acetylneuraminic acid (NANA), we determined hexose and NANA in the main spot of fraction G after preparative TLC purification. The molar ratio was found to be hexose:NANA, 3:1. Consequently, in order to identify the protein fraction in the proteolipid type complex, we determined iron and copper in the water phase derived from the extraction by Folch et al. (1957). The values for iron and copper were 0.43% and 0.34%, respectively, which confirmed the presence of red protein.

The rest of fraction G was further fractionated onto C_{18} HPLC column with the elution system described in Materials and Methods. The elution pattern is presented in Figure 3. Standard gangliosides eluted just after the solvent front until 19 min. All fractions were collected and tested for their ability to induce washed rabbit platelet aggregation or to inhibit PAF- and thrombin-induced aggregation.

We detected two fractions, namely, fractions 5 and 10 (Figure 3) that induced washed rabbit platelet aggregation. Both fractions caused aggregation with the same pattern as PAF, which is a rapid dose-dependent aggregation curve, reversible at low concentrations and irreversible at high concentrations. The EC_{50} values for fractions 5 and 10, namely, the fraction efficiency concentration that causes 50% aggregation, were 1.4×10^{-6} and 1.5×10^{-7} M, respectively, both on the basis of ester determination.

These aggregations were totally inhibited (100%) by BN 52021, a specific inhibitor of PAF-induced aggregation as well as of PAF binding in platelets. The enzymatic system of creatine phosphate/creatine phosphate kinase (CP/CPK) which is the specific inhibitor of ADP-induced aggregation, did not affect the aggregations induced by fractions 5 and 10. Additionally, indomethacine, the specific inhibitor of arachidonic induced aggregation, did not inhibit the above aggregations. An amount of PAF which induced platelet aggregation with a curve height equal with the ones induced by the fractions 5 and 10, was totally inhibited by the quantity of BN 52021 and not affected by the amounts of CP/CPK and indomethacine, used for the inhibition experiments of fractions 5 and 10.

Both fractions desensitized platelets against themselves and against PAF but not against thrombin. The results from the desensitization and cross-desensitization experiments as well as the ones with the specific inhibitors, demonstrate that these compounds act through receptors which are similar to the ones of PAF.

Treatment with human serum acetylhydrolase resulted to complete and rapid loss of the biological activity in both fractions. The hydrolysis had the same rate with the one observed when PAF, which induces aggregation curve of the same height with the examined samples, was used. Acetylhydrolase is an enzyme which specifically degrades acyl groups that consist of two to three carbon atoms, showing higher activity toward the acetyl-group. These results along with the existence of ester bonds demonstrate the presence of acetyl groups in the fractions 5 and 10.

Finally, the extraction method used and their recovery in the water phase of the Folch et al. method, the fact that these fractions migrated on TLC and on silica and C_{18} HPLC columns along with standard gangliosides as well as the results from the biological experiments and chemical determinations led to the conclusion that these compounds are *O*-acetyl derivatives of gangliosides.

Additionally, we detected a small number of fractions which were also eluted onto C_{18} HPLC column, in the region of gangliosides, and inhibited PAF- as well as

thrombin-induced aggregation. Their structures are under investigation.

In conclusion, it is the first time that the presence of gangliosides is reported from muscle fish. It is also interesting that we detected gangliosides which exhibited biological activity and some of them were identified as *O*-acetyl derivatives in which the acetyl group(s) are indispensible for the expression of their biological activity. The last finding is in accordance with the ones in literature which reported that gangliosides or their derivatives activate platelets or inhibit their activation (Ferroni et al., 1995; Tubaro et al., 1994).

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