Isolation, Characterization and Seasonal Variations in the Concentration of N^{ϵ} -(γ -Glutamyl)-lysine Isodi-peptide in the Blood Plasma of the Winter Flounder (*Pseudopleuronectes americanus*)

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A ninhydrin-positive compound was isolated from the plasma of the winter flounder (*Pseudopleuronectes americanus*) and identified as the isodi-peptide N^{ϵ} -(γ -glutamyl)-lysine. Wide seasonal variations in plasma concentration of this compound are found in the male flounder, whereas it is present only in trace amounts in the plasma of the female flounder throughout the year. In the male flounder the plasma concentration of N^{ϵ} -(γ -glutamyl)-lysine rises from January to a peak in May and June. It is during these latter months that the flounder spawn. After spawning, the plasma concentration of the isodi-peptide decreases to low basal values in October. These changes in plasma concentration of N^{ϵ} -(γ -glutamyl)-lysine may be related to changes in testes weight, spermatogenesis and spawning activity of the winter flounder.

A large number of γ -glutamyl isodi-peptides have been isolated from various biological sources. Human urine has been shown to contain γ -glutamylvaline, γ -glutamyl-leucine, γ -glutamylisoleucine and γ -glutamylornithine (Lou, 1975; Buchanan *et al.*, 1962). γ -Glutamylhistidine has been isolated from bovine brain (Kakimoto & Konishi, 1976), and these authors report that others have isolated γ -glutamylglutamine, γ -glutamylglutamic acid, γ -glutamylglycine, γ -glutamylalanine, γ -glutamylvaline, γ -glutamyl- β -aminoisobutyric acid, γ -glutamylisoleucine, S -methylglutathione and glutathione from mammalian brain. γ -Glutamylglutamic acid, γ -glutamylglutamine, γ -glutamylvaline, γ -glutamyl-leucine and γ -glutamylglutamylglutamic acid have been isolated from the broths of glutamic acid-fermenting bacteria (Hasegawa *et al.*, 1977). In addition, γ -glutamyltyrosine and γ -glutamylphenylalanine have been isolated from soya beans (Morris & Thompson, 1962).

The N^{ϵ} -(γ -glutamyl)-lysine cross-link has been the subject of a recent review (Folk & Finlayson, 1977). It has been shown to be a universal feature of certain structural proteins in all species investigated, and is found in proteins from plasma membranes, endoplasmic reticulum, skeletal muscle and cardiac muscle as well as in micro-organisms. The N^{ϵ} -(γ -glutamyl)-lysine cross-link stabilizes the structure

of fibrin clots, clotted vesicular protein, hair, quill and wool. However, to our knowledge, N^{ϵ} -(γ -glutamyl)-lysine isodi-peptide has been isolated only after digestion of proteins from these sources and has not previously been found in the free state in any biological material. In the present paper we report on the isolation and seasonal variations in the concentration of free and unbound N^{ϵ} -(γ -glutamyl)-lysine isodi-peptide in the blood plasma of the winter flounder (*Pseudopleuronectes americanus*).

Materials and Methods

Amino acid analysis

A total of 305 adult winter flounder (30–40 cm long) were taken between January 1975 and December 1976 from Chapel's Cove, Conception Bay, Newfoundland, by scuba divers using hand nets. Blood samples were taken either immediately after capture (field fish) or after holding the fish for 7 days in laboratory aquaria (laboratory fish) with circulating sea-water at ambient temperatures. Blood-sampling techniques and preparation of deproteinized plasma extracts were as previously reported (Squires *et al.*, 1979). Analysis of ninhydrin-positive substances was performed (i) by using a two-column sodium method with a Beckman model 120 Amino Acid Analyser (Beckman Instruments, Palo Alto, CA, U.S.A.) as previously described for amino acids (Squires *et al.*, 1976, 1979) or (ii) by using the Pico Buffer System IV and DC6A resin (Durrum

Abbreviations used; Cbz, benzyloxycarbonyl; Boc, *t*-butoxycarbonyl.

Instrument Corp., Sunnyvale, CA, U.S.A.) with the Beckman model 120 Amino Acid Analyser, or (iii) by using a single-column lithium method as described by Lee (1974) and modified in *Beckman Bulletin no. 121M TB-013* (July 1976), with a Beckman model 121M Amino Acid Analyser and Beckman Systems AA Computing Integrator for Amino Acid Analyser. In the older two-column sodium method (Squires *et al.*, 1976) N^{ϵ} -(γ -glutamyl)-lysine was eluted between methionine and isoleucine, whereas in both of the other methods N^{ϵ} -(γ -glutamyl)-lysine was eluted between leucine and tyrosine. All three methods of analysis were used during the study of the seasonal variations in plasma concentrations of N^{ϵ} -(γ -glutamyl)-lysine. In the subsequent work on the isolation and characterization of N^{ϵ} -(γ -glutamyl)-lysine, the modified method of Lee (1974) was employed. In this method, N^{ϵ} -(γ -glutamyl)-lysine was eluted at 85 min after sample injection (peak 85), as illustrated in Fig. 1.

Purification of peak 85

In the initial attempts at purification, deproteinized plasma extracts were applied to a 1.5 cm $\times$ 90 cm column of AG-50W X8 (200–400 mesh; H^+ form) ion-exchange resin (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and eluted with 2.5 M-HCl (Stein & Moore, 1949). Fractions were analysed for the presence of peak 85, and appropriate fractions were combined and concentrated by using a flash evaporator. During flash evaporation, the temperature of the heating bath was kept to a minimum, since N^{ϵ} -(γ -glutamyl)-lysine is subject to rearrangement reactions (Kornguth *et al.*, 1963). However, concentrations of peak 85 decreased by only 12% after 3 days in samples left at room temperature in 2 M-HCl. Fractions were repeatedly rechromatographed to obtain pure preparations of peak-85 material. Fractions enriched in peak 85 were also applied to a 1.5 cm $\times$ 90 cm column of Bio-Gel P-2 (Bio-Rad) and eluted with distilled water. Fractions containing peak 85 were combined and concentrated by using a flash evaporator. Subsequently, untreated plasma samples were rechromatographed on Bio-Gel P-2 to yield pure preparations of peak 85.

Hydrolysis techniques

Samples for acid hydrolysis (0.5 ml) were added to 0.5 ml of constant-boiling HCl (Pierce Chemical Co., Rockford, IL, U.S.A.) and heated at 110°C for 24 h. Hydrolysates were then dried in a vacuum oven over NaOH pellets, reconstituted with lithium citrate buffer, pH 2.2 (0.2 M in Li^+), and amino acid analyses were performed as described above.

Enzymic hydrolysis was attempted with a peptidase preparation (Sigma Chemical Co., St. Louis, MO, U.S.A.) that contained general proteolytic and

aminopeptidase activity. However, the preparation was heavily contaminated with free amino acids and, to remove these, 20 mg of the peptidase was dissolved in 1.0 ml of water and chromatographed on Bio-Gel P-2. Hydrolysis of a purified preparation of peak 85 was attempted with the purified enzyme preparation as per *Sigma Bulletin no. 251*. Hydrolysis of peak 85 was also attempted with γ -glutamyl transpeptidase (courtesy of N. Curthoys), purified to homogeneity by the method of Hughey & Curthoys (1976). Maleate was included in the incubation mixture in some experiments, since it has been reported (Tate & Meister, 1974) to stimulate the hydrolytic activity of γ -glutamyl transpeptidase.

High-voltage electrophoresis

High-voltage paper electrophoresis was performed with Whatman 3MM paper and a Savant flat-plate electrophoresis apparatus (Savant Instruments, Hicksville, NY U.S.A.). Electrophoretograms were run at pH 1.9 with 8% acetic acid and 2% formic acid and at pH 6.5 with 10% pyridine and 0.3% acetic acid. After being dried, electrophoretograms were developed with 1% ninhydrin in acetone. The mobility of the preparation of peak-85 material at pH 1.9 was calculated relative to serine and used in the estimation of molecular weight as described by Offord (1966).

Peptide synthesis

Various glutamyl-lysine dipeptides were synthesized from glutamic acid and lysine with appropriately blocked functional groups by coupling with dicyclohexylcarbodi-imide (Chaturvedi *et al.*, 1965) and de-blocking with HBr in acetic acid (Ben-Ishai, 1954).

N^{α} -(α -Glutamyl)-lysine synthesis. N^{ϵ} -Cbz-L-lysine benzyl ester hydrochloride (Sigma) (3 mmol) and triethylamine (3 mmol) were dissolved in 25 ml of acetonitrile, and N -Boc-L-glutamic acid γ -benzyl ester (Sigma) (3 mmol) was added with stirring. To this was added dropwise at 0°C 3 mmol of dicyclohexylcarbodi-imide in 10 ml of acetonitrile, and the mixture was stirred overnight at room temperature. Acetic acid (0.3 ml) was then added, the precipitated dicyclohexylurea filtered off, and the filtrate evaporated to dryness in a flash evaporator. Dry acetone (20 ml) was added, and the mixture was refluxed for 1–2 h, filtered while hot, and the filtrate dried *in vacuo*. The oil thus obtained was dissolved in 25 ml of acetic acid, and HBr gas was bubbled through the mixture at 80–85°C for 1 h. Anhydrous diethyl ether (75 ml) was added at 0°C, and the mixture was left overnight. The precipitate obtained was dissolved in a minimum amount of water and purified by chromatography on a 1.5 cm $\times$ 90 cm column of Bio-Gel P-2.

N^ε-(γ-Glutamyl)-lysine synthesis. *N*-Boc-L-glutamic acid α-benzyl ester (Sigma) and *N*^ε-Cbz-L-lysine benzyl ester hydrochloride were coupled and the dipeptide was de-blocked as described above for *N*^α-(α-glutamyl)-lysine.

N^ε-(γ-Glutamyl)-lysine: *N*^α-Cbz-L-lysine (3.6 mmol) and toluene-*p*-sulphonic acid (3.6 mmol) were refluxed for 2 h in 10 ml of dry methanol with low heat and left overnight at room temperature. Low-boiling components (7 ml) were then distilled off, excess dry methanol was added, and a further 3 ml was distilled off. The distillate was then concentrated on a flash evaporator to yield a clear oil. Toluene-*p*-sulphonic acid (1 mmol), dry benzyl alcohol (10 ml) and dry benzene (5 ml) were then added, and the mixture was refluxed with gentle heat for 3 h. Low-boiling components were distilled off under N₂, and excess ether was added to give a clear oily precipitate. This was dissolved in 25 ml of acetonitrile with triethylamine (3 mmol) and *N*-Boc-glutamic acid α-benzyl ester (3.5 mmol), and dicyclohexylcarbodi-imide (3.5 mol) in 10 ml of acetonitrile was added dropwise at 0°C. The

reaction mixture was left overnight at 25°C and then worked up as described for *N*^α-(α-glutamyl)-lysine to yield *N*^ε-(γ-glutamyl)-lysine.

Results

Seasonal changes in plasma concentrations of peak-85 material

During the course of a study on the seasonal changes in plasma free amino acids in the winter flounder (Squires *et al.*, 1979), a large unknown peak appeared on the amino acid analyser chromatograms at 85 min after injection (peak 85, Fig. 1). The size of this peak varied throughout the year, and the annual profile of changes in plasma concentrations of peak-85 material is depicted in Fig. 2. Male and female flounders were sampled at intervals from January 1975 to December 1976; however, results obtained from fish sampled in 1975 and 1976 were combined, since statistical analysis showed no significant differences in profiles from either year. Groups of samples taken over a period of a few weeks that were not statistically different

Fig. 1. Separation of amino acids and glutamyl-lysine isodi-peptides in the amino acid analyser. Percentage transmittance was recorded at 570 nm (—) and 440 nm (---). A standard calibration mixture of amino acids (250 nmol/ml) containing the synthesized glutamyl-lysine isodi-peptides was analysed as described in the text (method iii). The three γ-glutamyl-lysine isodi-peptides were resolved into distinct peaks (see insert).

Fig. 2. Changes in plasma concentrations of N^ϵ -(γ -glutamyl)-lysine isopeptide in the winter flounder. Plasma concentrations of N^ϵ -(γ -glutamyl)-lysine isopeptide were measured as described in the Materials and Methods section for male flounders (a) and female flounders (b). Samples taken over a 1–2-week period that were not significantly different were combined and plotted with use of an average sampling date for both laboratory fish (----) and field fish (—). Vertical bars represent 1 S.E.M. and the numbers in parentheses refer to the numbers of fish sampled.

were combined and plotted with use of an average sampling date.

Quite different profiles were obtained in plasma concentrations of peak-85 material in male and female winter flounders. Only trace amounts of peak-85 material were present in the plasma of both laboratory and field female flounders sampled throughout the year. However, greater concentrations of peak-85 material are evident in the plasma of both laboratory and field male flounders. In the male field fish, there was a steady rise in plasma concentration of peak-85 material from January to May followed by a steady decrease to low basal values in October. In the male laboratory fish, plasma concentrations of peak-85 material rose to a plateau value from January through to September and declined sharply in October to remain low until December.

Characterization of peak-85 material

The molecular weight of the peak-85 material was determined by using gel-filtration chromatography. Peptide markers of known molecular weight were applied to a 1.5 cm $\times$ 90 cm column of Bio-Gel P-2 and eluted with water at a flow rate of 1–2 ml/min. K_{av} values for these compounds were calculated as $(V_e - V_0)/(V_t - V_0)$ where V_e , V_0 and V_t are the elution volume of the peptide, void volume of the column as determined with Blue Dextran and the total volume of the gel bed respectively (Reiland, 1971). A linear regression analysis of a plot of K_{av} values for each compound versus the logarithm of their molecular weights gave a correlation coefficient (r^2) value of 0.988 and a calculated molecular weight for the peak-85 material of 260.8 ± 0.3 . This compares with a calculated molecular weight for N^ϵ -(γ -glutamyl)-lysine of 275.3.

Acid hydrolysis was carried out on a number of purified preparations of peak-85 material. Glutamic acid and lysine were present in equimolar amounts, with 7% aspartic acid and threonine and 3% tyrosine present as contaminants. Attempts at enzymic hydrolysis of peak-85 peptide with purified peptidase as described in the Materials and Methods section proved to be futile and suggested that the peak-85 peptide was not an α -linked peptide.

High-voltage electrophoresis of purified peak-85 peptide and hydrolysates was performed at pH 1.9 and 6.5. The results confirmed that peak-85 peptide was composed of lysine and glutamic acid. The mobility of peak-85 peptide at pH 1.9 relative to serine was 1.07, which according to Offord (1966) corresponds to a molecular weight of approx. 250 for a peptide having a net charge of ± 1 and 800 for a peptide having a net charge of ± 2 . At pH 6.5, the peak-85 peptide did not migrate from the origin and was thus uncharged, indicating that glutamic acid and not glutamine was present in the peptide.

The presence of a γ -glutamyl link in the peak-85 peptide was confirmed by enzymic hydrolysis with purified γ -glutamyl transpeptidase (Hughey & Curthoys, 1976; Tate & Meister, 1974). The preparation was not contaminated with α -peptidases, since it had only 0.5% of the hydrolytic activity towards glycylglycine as it had towards the peak-85 peptide. Addition of 50 mM-maleate in the incubation mixture gave a 5–6-fold stimulation of hydrolysis of peak-85 peptide to yield lysine and glutamic acid in equimolar amounts.

At this point, N^ϵ -(γ -glutamyl)-lysine was synthesized as described in the Materials and Methods section. This compound was eluted at 79 min after injection on the amino acid chromatogram (Fig. 1) and co-eluted with isoleucine. A mixture of equal parts of authentic peak-85 peptide and N^ϵ -(γ -glutamyl)-lysine was resolved by the amino acid analyser into two distinct peaks, indicating that

N^α-(γ-glutamyl)-lysine and the peak-85 peptide were different. N^ε-(γ-Glutamyl)-lysine was then synthesized as described. This compound was eluted at 85 min after injection with the peak-85 peptide (Fig. 1). Addition of the synthesized N^ε-(γ-glutamyl)-lysine preparation to a male-flounder plasma extract gave a calculated increase in peak-85 peptide (Fig. 3). Acid hydrolysis of the N^ε-(γ-glutamyl)-lysine

preparation gave equimolar amounts of glutamic acid and lysine.

Discussion

N^ε-(γ-Glutamyl)-lysine isodipeptide is thus present unbound in the plasma of the winter flounder. In addition, high and variable concentrations are found in the plasma of the male flounder whereas the isodipeptide is found only in trace amounts in the blood plasma of the female flounder. These observations lead us to assume that N^ε-(γ-glutamyl)-lysine is in some way involved in the reproductive cycle of the male flounder. In this regard, it is instructive to compare plasma concentrations of N^ε-(γ-glutamyl)-lysine in the male flounder to testis weight at different times of the year (Fletcher & King, 1978). After spawning in June, the male flounder regenerates the testes, and testes weights reach a peak in November. At this time, concentrations of N^ε-(γ-glutamyl)-lysine in the plasma are low. From December to May, testes weights decrease slightly, and the fish are, for the latter months, awaiting spawning. During this time, N^ε-(γ-glutamyl)-lysine concentrations increase in the plasma and decrease sharply in July–August after spawning. The correlation that is evident here suggests that N^ε-(γ-glutamyl)-lysine formation is in some way related to spermatogenesis or in the initiation of spawning activity in the winter flounder.

The possibility exists that N^ε-(γ-glutamyl)-lysine may be formed as a result of tissue breakdown during the winter months. It has been shown by Waibel & Carpenter (1972) that N^ε-(γ-glutamyl)-lysine is poorly metabolized whereas simple α-linked dipeptides are rapidly removed (Adibi & Krzyzisk, 1977) when injected into the bloodstream of the rat. Thus degradation of tissue during the winter months may cause the release of N^ε-(γ-glutamyl)-lysine isodipeptide into the bloodstream. However, this does not explain why the isodipeptide is not found in the plasma of the female winter flounder.

N^ε-(γ-Glutamyl)-lysine may instead be the product of the activation of a peptide hormone, or may in itself play a hormonal role. It is noteworthy that a tripeptide has been isolated that stimulates DNA, RNA and protein synthesis in rat liver cells (Pickard & Thaler, 1973) when present in nanomolar concentrations. It may therefore be possible that the isodipeptide N^ε-(γ-glutamyl)-lysine may be important in stimulating maturation of the testes in the winter flounder. Alternatively, the isodipeptide may be important as an attractant to female winter flounder, thus stimulating pair formation as an aid to successful spawning. Little work has been done on the chemical nature of sex pheromones in fish; however, it has been shown (Sutterlin, 1975) that the

Fig. 3. Co-chromatography of peak-85 peptide with synthetic N^ε-(γ-glutamyl)-lysine isodipeptide

A sample of male-flounder plasma was deproteinized and analysed for ninhydrin-positive substances as described in the Materials and Methods section (method iii). Percentage transmittance was recorded at 570 nm. N^ε-(γ-Glutamyl)-lysine was present in the diluted plasma extract at 76.4 nmol/ml as leucine equivalents (—). Synthetic N^ε-(γ-glutamyl)-lysine was added to the plasma extract at a final concentration of 497.7 nmol/ml as leucine equivalents and the sample was rechromatographed (---). The concentration of peak-85 peptide rose to 558.3 nmol/ml as leucine equivalents, which corresponds to 97.2% of the expected value.

winter flounder is attracted to amino acids in low concentrations.

The possibility also exists that N^ϵ -(γ -glutamyl)-lysine is a product of the spermatogenic cycle. It has been reported (Hagenmaier *et al.*, 1976) that the chorions of fertilized eggs of the rainbow trout (*Salmo gairdnerii*) contain the N^ϵ -(γ -glutamyl)-lysine cross-linkage whereas the chorions of unfertilized eggs do not. Presumably, then, the semen of the rainbow trout contains or initiates the production of a transamidase enzyme that catalyses the formation of N^ϵ -(γ -glutamyl)-lysine bonds in the chorions of the fertilized eggs. In this regard, it has been shown that a transamidase reaction is involved in the coagulation of guinea-pig vesicular protein (Williams-Ashman *et al.*, 1972). The possible presence of a transamidase enzyme in the semen of the winter flounder may well account for the presence of N^ϵ -(γ -glutamyl)-lysine isodipeptide in the blood plasma. The concentration of N^ϵ -(γ -glutamyl)-lysine isodipeptide in the plasma may well be useful as an index of sexual maturity in the winter flounder.

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