ORIGINAL RESEARCH

Megalophthalmia in Black Moor goldfish: an experimental model to study metabolic events associated with eye expansion

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Abstract

Aim: To compare the biochemical contents in the vitreous of Black Moor and common goldfish in order to explore some of the biochemical changes associated with the unlimited eye expansion in Black Moor goldfish.

Materials and methods: Vitreous humor was analyzed for small molecules and macromolecules. To separate small molecules, a Delta PAK C_{18} column was eluted by acetonitrile-trifluoroacetic acid-water, 2:0.01:97.99. To separate peptides, a Delta PAK C_4 column was eluted by increasing acetonitrile concentration from 20% to 80%. The eluant was monitored by a photo diode array detector and platform LCZ mass detector. The chemical structure of compound 204 was investigated by mass spectrometry and nuclear magnetic resonance spectroscopy.

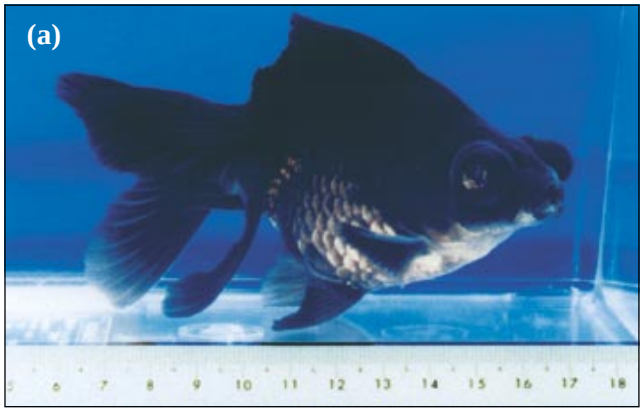
Results: Three remarkable biochemical properties were observed in the vitreous of Black Moor goldfish, including a marked elevation of lactic acid, the occurrence of compound 204, a dicarboxylic carbohydrate with a 6-member lactone ring structure, and a high concentration of peptides 13.67K and 27.34K.

Conclusion: A comparison of the biochemical contents of the vitreous between Black Moor and common goldfish revealed specific metabolic changes associated with eye growth. The high lactic acid content indicates the activation of glycolysis when the normal restriction of eye growth after birth is lost. The occurrence of compound 204 reveals a new metabolic pathway of glucose metabolism in fishes not seen in mammalian tissues. The high amount of peptides 13.67K and 27.34K indicates a marked change in the biosynthetic activity for peptides in the vitreous cavity of growing eyes. The present data demonstrate the value of Black Moor goldfish as a model to study many biochemical events associated with eye growth.

Key words: Carbohydrates, Goldfish, Glycolysis, Lactic acid, Lactones, Magnetic resonance spectroscopy, Spectrum analysis, mass

Introduction

Myopia is a prevalent problem in Hong Kong. Both hereditary and environmental factors affect the refractive status of human eyes.[1-3] Epidemiological studies indicate that the amount of 'near-work', or 'blur vision' accounts for the



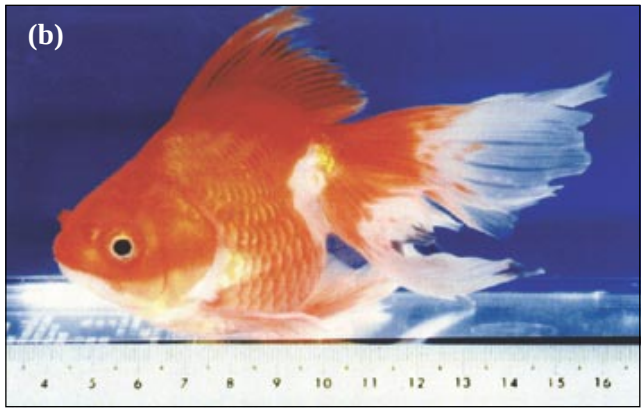


Figure 1. (a) Black Moor goldfish; (b) common goldfish.

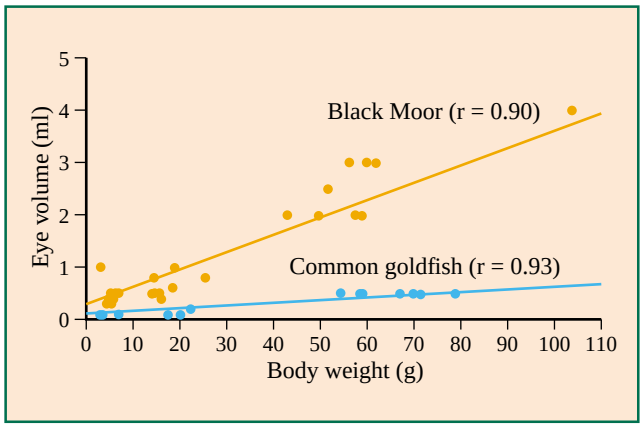


Figure 2. Correlation of eye volume to body weight of Black Moor goldfish (orange circles) and common goldfishes (blue circles).

difference in refractive errors among some children of the same genetic background.[4,5] The effect of environmental factors on the refractive status of human eyes have been demonstrated in animal models.[6-14]

A common biological feature of myopia in human and animal models is expansion of the affected eyeball. Usually, normal eye growth markedly slows down at birth and stops before maturity. The mechanism controlling the eye size is an important topic in myopia research. Three types of animal models have been subjected to investigation for biochemical events associated with eye expansion: mice, rabbits, and Black Moor goldfish. Zhou and Williams demonstrated the involvement of numerous genes in controlling the eye size by comparing different strains of mice with different eye sizes.[15] An abnormal enlargement of the eye after birth concurrent with elevated intraocular pressure has been reported in hereditary mutant rabbits.[16-19] The high intraocular pressure in buphthalmic rabbits causes corneal edema, and loss of blood-aqueous barrier as their eyes expand.[18,19] Biochemical changes in the intraocular fluid of buphthalmic rabbits reveals tissue damage caused by elevated intraocular pressure.

The eyes of Black Moor goldfish have normal intraocular pressure, yet their eyes grow indefinitely after birth resulting in enlargement and protrusion of the strongly myopic eyes.[20-22] The eye sizes of common goldfish remain within a small range after birth (**Figures 1** and **2**). The continuously growing eyes of Black Moor goldfish involve the expansion of the sclera, the retina, and possibly the vitreous gel structure. It could be possible that some of the molecular events associated with eye growth, particularly the metabolites released from the retina, and changes in the vitreous gel structure, may be elucidated from biochemical properties of the vitreous humor. Obviously, investigation of the sclera and retina are important to fully understand the diversity of events associated with eye growth. The vitreous humor is a simple tissue, easily obtained for investigation. Therefore, our initial study is limited to investigation of the vitreous humor. This report summarizes our initial findings on the biochemical difference in the vitreous humor between Black Moor and common goldfish. The significance of these findings and promising future research directions are discussed.

Materials and methods

Both Black Moor and common goldfish were obtained from Hoipei aquarium, Hong Kong. The goldfish were anaesthetized by being immersed in 0.05% 3-aminobenzoic acid ethyl ester (Sigma, St Louis, USA). The eye was rapidly dissected from the eye socket, dried on a piece of absorbent paper, then immersed in a graduated cylinder containing cold phosphate-buffered saline to estimate the eye volume. The eye was placed on an absorbent paper again. The vitreous was aspirated by puncturing the cornea with a 25-gauge needle connected to a tuberculin syringe. Specimens were stored at -80°C.

Chromatographic examination of small molecules

Delta PAK C_{18} column was equilibrated in acetonitrile-water-trifluoroacetic acid (2.00:97.99:0.01) and eluted by the same solvent at 0.5 ml/minutes. The eluant from the column was delivered to a photo diode array detector, then to a mass detector (Micromass Platform LCZ, Waters Associates, Medford, USA) through a fine tubing (inside diameter, 0.005 inches). An aliquot of each specimen was injected into a Delta PAK C_{18} column. The optimized settings in the mass detector were: nitrogen gas flow, 500 L/hour; capillary voltage, 2.8 kV; cone voltage, 10 V; source temperature, 140°C; and desolvation temperature,

350°C. All mass spectra were recorded under a full scan operation for both positive and negative ions, with a scan range from m/z 20 to 600. The amount of lactic acid in the eluant was calculated from the selected-ion recording mode that monitored the ion current produced from the deprotonated molecular ion (m/z = 89) of lactic acid. The peak area of the lactic acid peak was used to calculate the lactic acid concentration by comparing with the peak area of a standard lactic acid solution analyzed in the same manner.

Chromatographic examination of peptides

Delta PAK C_4 column was also used for peptide analysis. The column was equilibrated in 20% acetonitrile:80% water. Formic acid was added to the elution solvent to a final concentration of 0.02 M. The concentration of acetonitrile increased linearly to 30% at 10 minutes, to 50% at 70 minutes, and to 80% at 90 minutes. Formic acid was kept constant at 0.02 M throughout the analyses. The eluant was passed through the photodiode array detector, and mass detector. The optimized settings in the mass detector were: nitrogen gas flow, 350 L/hour; capillary voltage, 3.5 kV; cone voltage, 10 V; source temperature, 140°C, and desolvation temperature, 350°C. To monitor peptides eluted from the column, the eluant was first analyzed by the photo diode array detector, 210 nm, followed by a mass detector. The mass chromatograms were obtained by electrospray for positive ions, m/z 500 to 3000. Each peak was processed for ion sizes by MaxEnt software as shown in **Figures 3** and **4**.

Nuclear magnetic resonance spectroscopy

The vitreous humor obtained from Black Moor goldfish was subjected to chromatography on a μ-Bondapak-NH_2 column as previously described.[24] The fractions containing the major compound detected by ultraviolet (UV) absorption

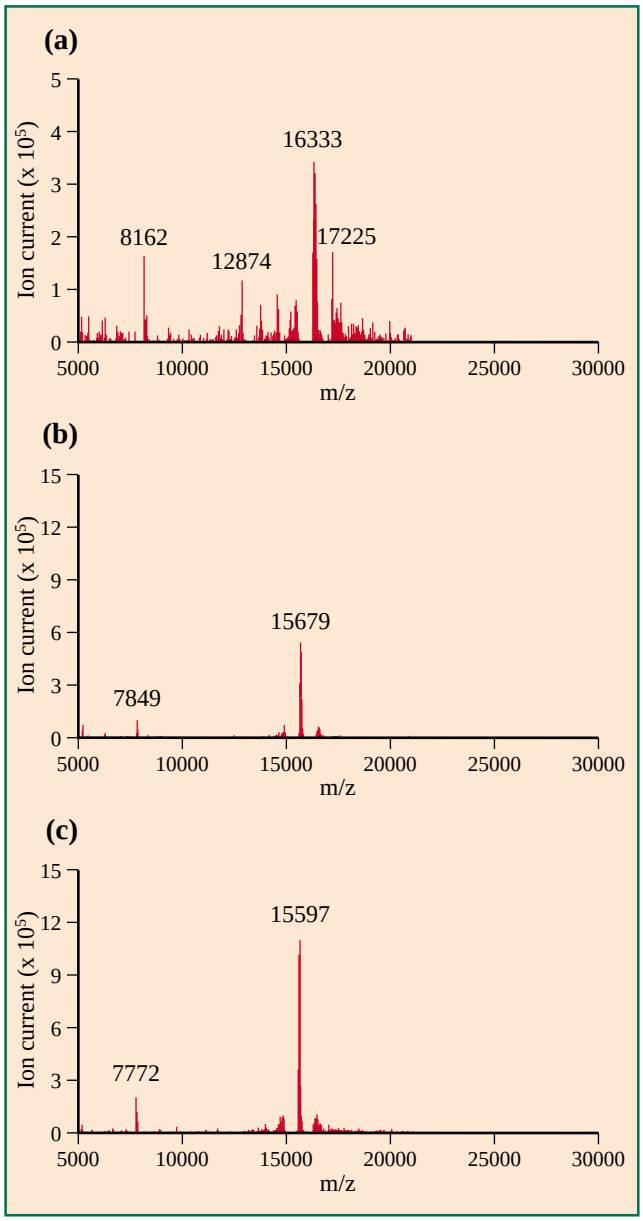


Figure 3. Ion size of the major peaks in common goldfish. (a) Peak 7; (b) peak 9; and (c) peak 10.

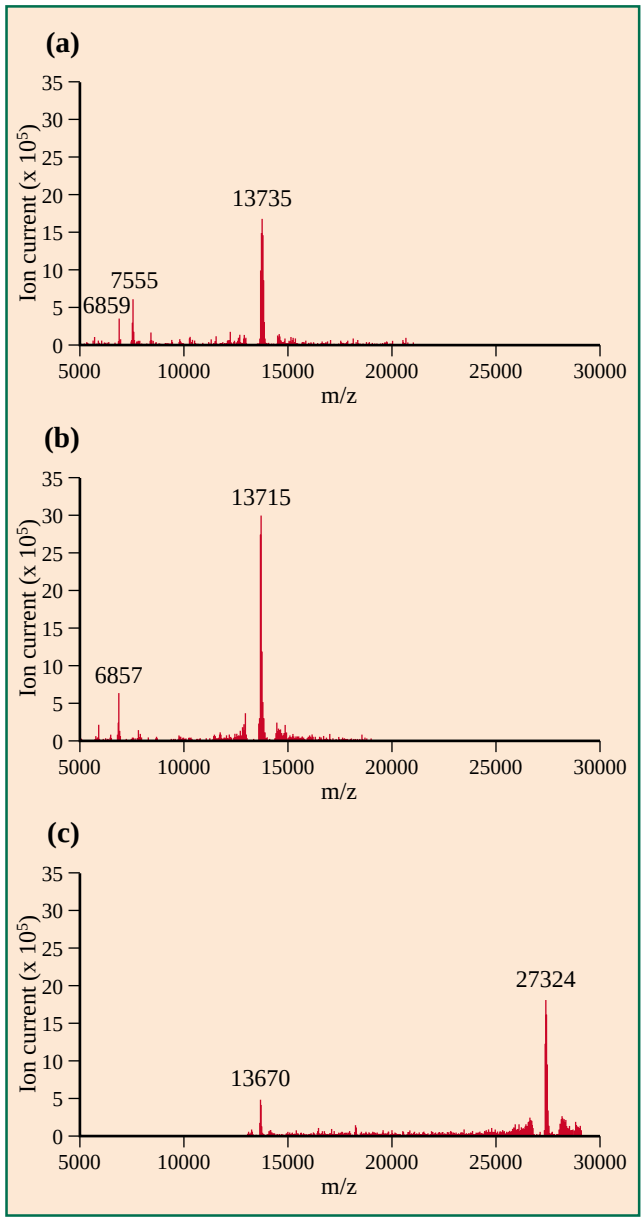


Figure 4. Ion size of the major peaks in Black Moor goldfish. (a) Peak 5; (b) peak 6; and (c) peak 8.

(290 nm) was lyophilized and subjected to chromatography on a Delta PAK C_{18} column as shown in **Figure 5**. The fraction containing the major peak was combined, lyophilized, and sent to the NMR Analysis and Solution laboratory (Decatur, USA) for nuclear magnetic resonance (NMR) analysis.

Results

Compound 204

Purification of compound 204 from the vitreous of Black Moor goldfish

We have recently reported a unique difference between the chromatograms of aqueous humor obtained from normal and Black Moor goldfish. The chromatogram of the aqueous humor from Black Moor goldfish contained a major peak with a UV absorption maximum at 290 nm. This peak was not seen in the aqueous humor of common goldfish.[24] The same results were observed when the vitreous humor of the 2 species were compared. In previous studies, specimens of aqueous humor were injected to a μ-Bondapak-NH_2 column, eluted by 10 mM ammonium phosphate.[18,19] We have used the previous method to isolate a large amount of the unknown compound from the vitreous humor for chemical structure characterization by mass spectrometry and nuclear magnetic resonance spectroscopy. Since ammonium phosphate interferes with mass spectrometric analysis, compound 204 isolated by the method previously described[24] was subjected to chromatography on a Delta PAK C_{18} column to remove ammonium phosphate. Only 1 peak was observed (**Figure 5a**). However, the UV absorption maximum of the compound was 270 nm (**Figure 5b**), different from our previously reported value of 290 nm. The change in UV absorption maximum from 290 nm to 270 nm of the same compound was due to the effect of different solvents used in the 2 chromatographic procedures. We have confirmed the effect of solvent on the UV absorption maximum by a comparison of the absorption spectrum of the same specimen in 2 different solvents. The peak collected from the Delta PAK C_{18} column had an absorption maximum at 270 nm. When this was diluted with 10 mM ammonium phosphate, the absorption maximum changed to 290 nm.

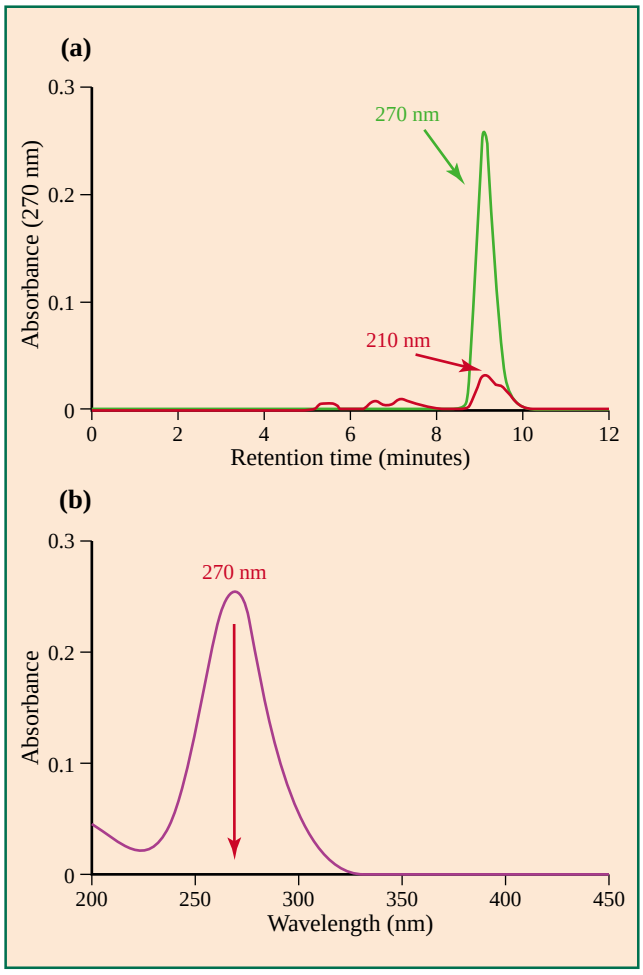


Figure 5. Purification of compound 204 from vitreous. (a) Compound 204 isolated from μ-Bondapak-NH_2 column was injected into a Delta PAK C_{18} column and eluted by 2% acetonitrile as described in the text. (b) Light absorption spectrum of the major peak is shown in (a).

Ion size of compound 204 determined by mass spectrometry

Electrospray analysis for protonated ions showed a major band of m/z 205 and a minor band of m/z 187 (**Figure 6a**). Many carboxylic compounds undergo fragmentation in the mass detector, producing a smaller fragment due to the loss of H_2O. The ion m/z 187 must be the fragment of 205 after the loss of H_2O. Electrospray analysis for deprotonated ions showed only one band, m/z 203 (**Figure 6b**). Therefore, the molecular size of the unknown must be 204. We now designate the unknown as compound 204.

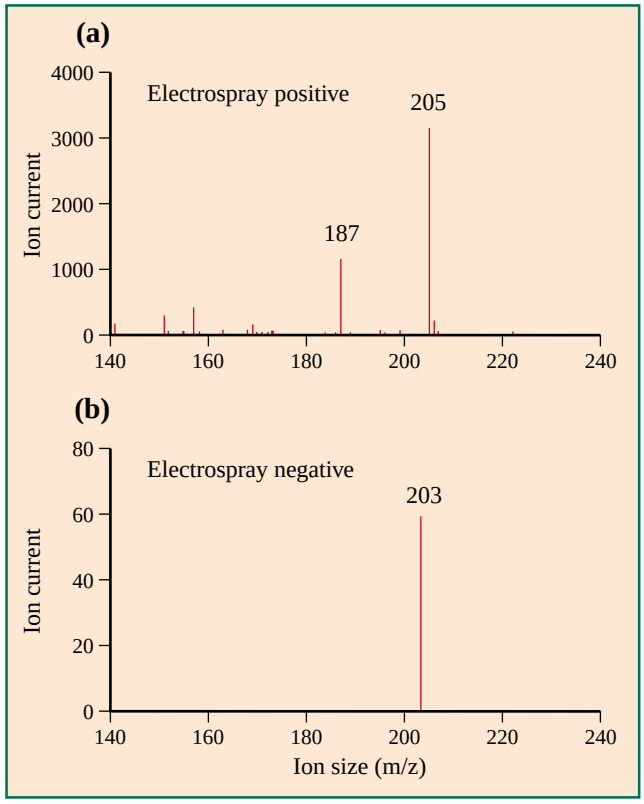


Figure 6. Molecular size of compound 204. (a) The positive charged ion size; (b) the negative charged ion size.

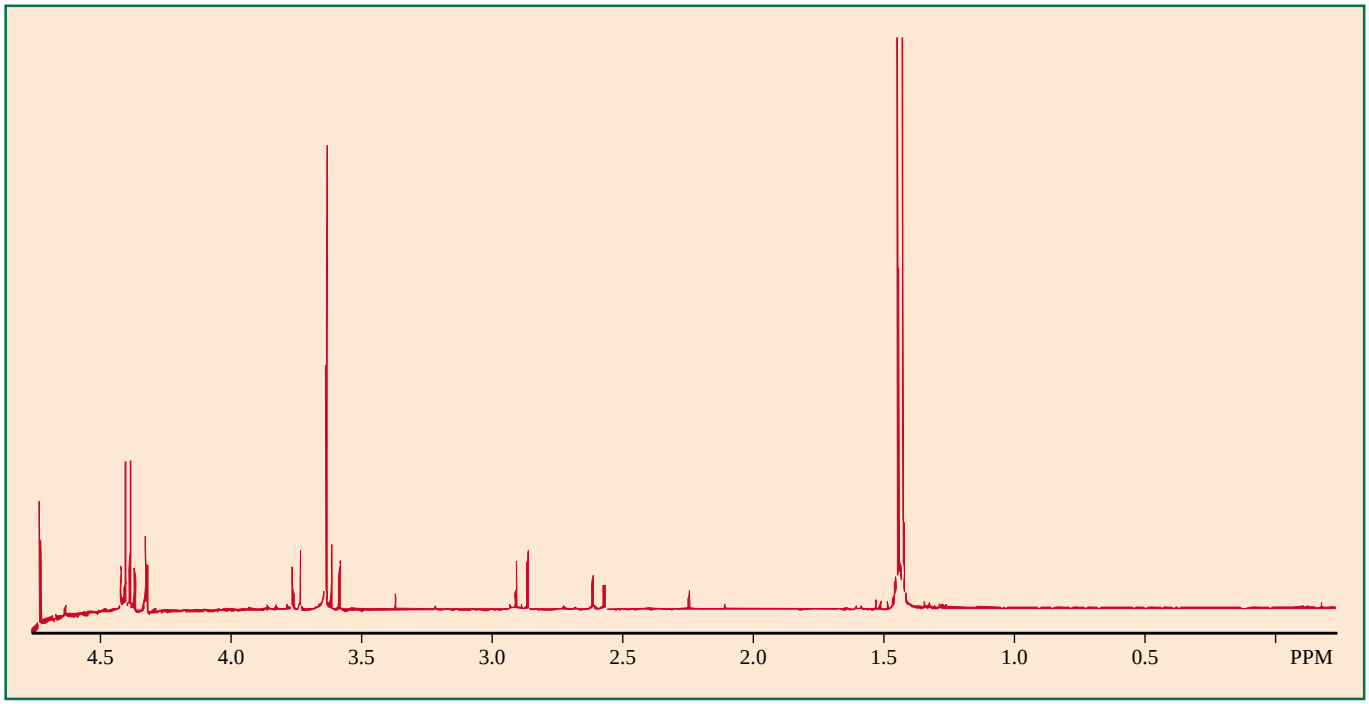


Figure 7. Nuclear magnetic resonance spectrum of compound 204.

Nuclear magnetic resonance spectroscopy of compound 204

The NMR spectroscopy data (**Figure 7**) indicates the presence of 2 compounds. The resonance between 1.4 to 1.5 ppm is the characteristic of lactic acid. The rest of the resonance spectrum indicates a compound with -CH_2-COOH linked to a dihydroxy, lactone ring as shown in **Figure 8**.

The coinciding location of the 270 nm absorption peak to the m/z 203 peak in the chromatogram confirmed that compound 204 has the UV absorption maximum at 270 nm (**Figure 9**). Lactic acid, m/z 89, was eluted from the column behind compound 204. When a small amount of vitreous was applied to the column, compound 204 and lactic acid were well separated as shown in **Figure 9**. In our isolation procedure, a large amount of the specimen was applied to the column. It was expected that some lactic acid was present in compound 204 prepared for NMR spectroscopy analysis.

Lactic acid content in the vitreous of Black Moor and common goldfish

The presence of lactic acid in the vitreous indicated by NMR spectroscopy, led us to examine if there were differences in

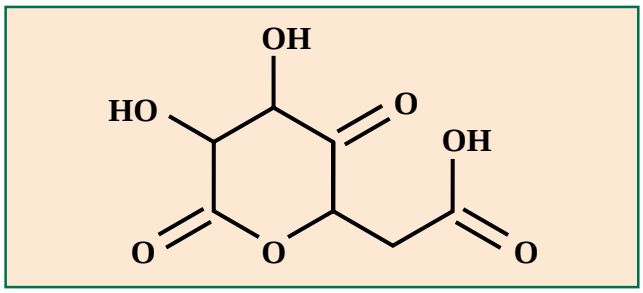


Figure 8. A compound with -CH_2-COOH linked to a dihydroxy, lactone ring.

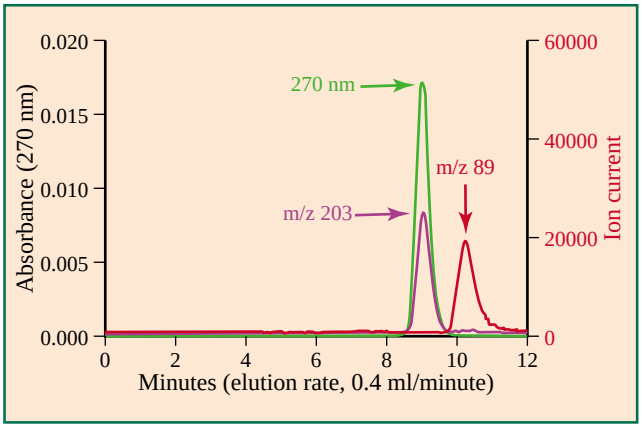


Figure 9. Chromatogram of aqueous from Black Moor goldfish. Compound 204 detected by 270 nm absorbance (purple line) coincides with compound 204 detected by the deprotonated ion m/z 203 (green line) and is separated from lactic acid detected by deprotonated ion m/z 89 (red line).

lactic acid content between the vitreous of Black Moor and common goldfish. The vitreous humor occupies the major volume of an eye. We estimated the total lactic acid per eye by multiplying the concentration of lactic acid in the vitreous humor with the total eye volume, assuming that lactic acid concentration is approximately the same throughout the eye.

Among Black Moor goldfish, the eye volume increased as the fish grew in weight (**Figure 2**, orange circles). The lactic acid per eye also increased in proportion to the body weight (**Figure 10**, orange circles). The eye volumes of the common goldfish fell within a narrow range (**Figure 2**, blue circles), approaching a constant volume as the body size increased to 20 g (**Figure 2**, blue circles). The lactic acid per eye also remained approximately the same among different

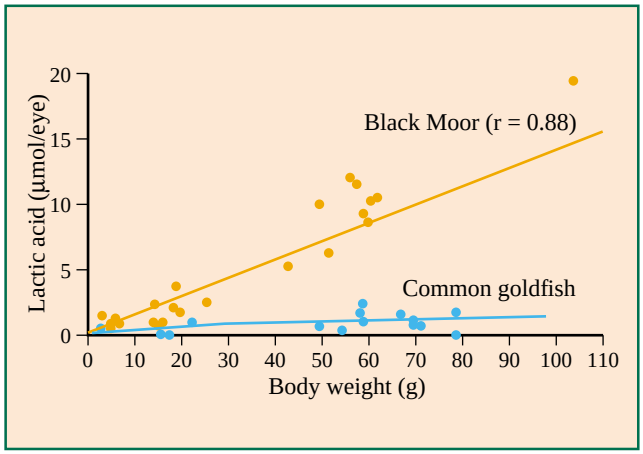


Figure 10. Correlation of lactic acid (µmol/eye) to body weight of Black Moor goldfish (orange circles) and common goldfish (blue circles).

sizes of common goldfish (**Figure 10**, blue circles). The amount of lactic acid in the eye of Black Moor goldfish was higher than that in the common goldfish.

Peptides in the vitreous of Black Moor and common goldfish

The Delta PAK C_4 column was used for the examination of peptides in the vitreous. A high concentration of acetonitrile was needed to elute peptides from the column. The chromatograms showed much higher amounts of peptides in the vitreous of Black Moor goldfish (**Figure 11**, black line) than that of common goldfish (**Figure 11**, red line).

The peptides in the vitreous of Black Moor goldfish were divided into 8 peaks based on their retention time on the column. Small molecules were separated on the column eluted by low acetonitrile concentration (2%), as shown in **Figure 5a**. When the peptides were examined, the column was eluted by an initial concentration of 20% acetonitrile. The high concentration of acetonitrile eluted many small molecules together as one sharp peak (**Figure 11**, peak 1). Albumin was eluted at the retention time of 33 minutes. Peaks 2, 4, and 7 contained a heterogeneous mixture of peptides not well resolved. It is difficult to characterize peaks 1 to 4.

The major peptides in the vitreous of common goldfish are hydrophobic, eluted from the column after the acetonitrile concentration is increased to 80% (**Figure 11**, red line). Their molecular sizes are approximately the same (**Figures 3a** to **c**). Peaks 9 and 10 of common goldfish are absent in Black Moor goldfish.

The most interesting peaks were 6 and 8 of Black Moor goldfish (**Figure 11**). Each of these 2 peaks contains a major and minor peptide. The major peptide in peak 8 (**Figure 4c**) has a molecular size twice of that of peak 6 (**Figure 4b**). A very small amount of peak 6 was observed in common goldfish. Peak 8 was absent in the vitreous of common goldfish. The major peptide in peak 5 (**Figure 4a**) has a molecular size very similar to that in peak 6.

Discussion

The rapid formation of the eye through the interaction of the ectoderm and the forebrain during embryonic development is fascinating. Although we cannot find a report on the embryonic development of fish eye, thorough investigations have been reported in other species. In chick embryo, the formation of the eye cup is completed on day 2 of incubation and the eye radius expands rapidly, approaching the maximal size on day 10. The growth of the body

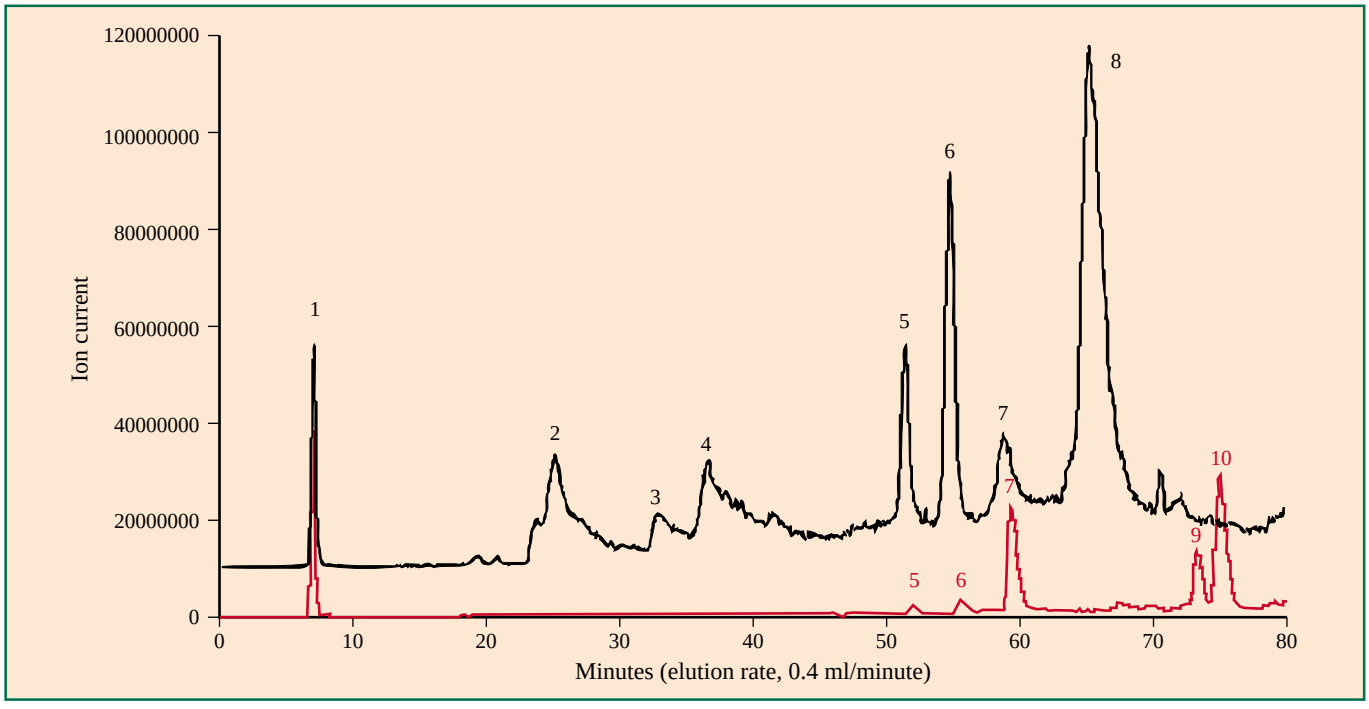


Figure 11. Peptides in vitreous of Black Moor goldfish (black line) and common goldfish (red line).

accelerates in the remaining incubation period with little change in the eye size up to day 18, the time of hatching.[25-26] An appropriate intraocular pressure is important to allow a rapid expansion of the eye during the early stage of the embryonic development. Loss of intraocular pressure by surgical removal of the lens in chick embryo[27,28] or genetically faulty lens development in the mouse embryo[29] leads to micro-ophthalmia.

While the rapid expansion of the eye by intraocular pressure can be explained as discussed above, the mechanism that restricts the increase of the eye size after birth is not known. Similar to many mammalian eyes, the eyes of common goldfish grow slowly after birth (**Figure 2**, blue circles). The eyes of Black Moor goldfish grow continuously in proportion to their body sizes (**Figure 2,** orange circles). The eye expansion of Black Moor goldfish reflects a loss in genetic control on eye growth seen among common goldfish and many other species. Megalophthalmia in Black Moor goldfish provides a good model to investigate metabolic events leading to a continuous expansion of an eye after birth.[30]

We have previously reported a remarkable chromatographic difference between the aqueous humor of Black Moor goldfish and that of common goldfish.[24] In many mammalian eyes, the major optically active compound in intraocular fluid is ascorbic acid.[23] There is very little ascorbic acid in the intraocular fluid of nocturnal animals.[23] The vitreous and aqueous humor of common goldfish is similar to that of nocturnal animals. There is negligible ascorbic or compound 204 detectable in the light absorption chromatogram of aqueous humor and vitreous of common goldfish. However, the vitreous and aqueous humor of Black Moor goldfish have a remarkably high amount of a compound with a unique UV absorption spectrum, as shown in **Figure 5b**. We now have characterized the unknown compound as a lactone with a molecular size of 204 by mass spectrometry and nuclear magnetic resonance spectroscopy. The presence of lactic acid in our preparation of compound 204 led us to observe the remarkable elevation of lactic acid in the eyes of Black Moor goldfish. We further compared the peptides in the vitreous of Black Moor and common goldfish and observed the marked elevation of peptides 13.67K and 27.29K in the vitreous of Black Moor goldfish. The significance of compound 204, lactic acid, and peptides are discussed as follows.

Compound 204

Among the small metabolites in intraocular fluid, the remarkably high amounts of compound 204 caught our initial attention.[24] This compound appears as the major small metabolite noticeable in the chromatogram of the vitreous and aqueous humor of small Black Moor goldfish. It has a unique UV absorption spectrum, A_{max} equals 270 nm in acetonitrile-trifluoroacetic acid and 290 nm in ammonium phosphate. It has no absorption at short wavelength (<230 nm). This light absorption characteristic indicates a structure of carbohydrate and rules out the structure of aromatic amino acid and nucleic acid. NMR spectroscopy reveals a structure of a carbohydrate with a lactone structure similar to that of ascorbic acid. The proposed structure meets the properties indicated from mass spectrometry and NMR spectroscopy data.

The proposed structure has a similarity to that of ascorbic acid. Ascorbic acid has 6 carbons with a 5-member lactone ring structure instead of 7 carbons and a 6-member lactone ring in compound 204. The only known carbohydrate metabolite with 7 carbons is sedoheptulose, which is formed through transketolase and transaldolase reactions in the pentose phosphate pathway. We have observed a large amount of compound 204 in the blood of 5 different species of goldfish. A large amount of this compound in intraocular fluid has been observed only in Black Moor goldfish. Since this compound also occurs in fish blood, our study has revealed a new carbohydrate metabolism in fish not seen in mammalian tissues. Future investigation of the metabolic pathway leading to the formation and degradation of this compound will be useful to elucidate its role in eye growth.

Lactic acid in vitreous humor

Continuous eye growth involves many different metabolic activities. All metabolic activities derive energy directly or indirectly from glucose through the Myerhoff pathway. In the absence of oxygen, glucose is metabolized to lactic acid, known as anaerobic glycolysis. The retina has the highest anaerobic glycolysis rate among all tissues analysed.[31] Since glycolysis supplies the energy requirement for different metabolic activities in the retina, one expects that the increase in glycolysis is more obvious than the increase of each individual metabolic activity involved in a growing eye. Among the common goldfish, there is little eye growth after birth, and there is little change in intraocular lactic acid (**Figure 10**). The common goldfish has a mechanism to control eye growth after birth concurrent with a constant glycolysis rate. The Black Moor goldfish looses the mechanism to restrict their eye growth, and glycolysis.

In this report, we present the total lactic acid per eye (**Figure 10**). In order to fill a large eye with a specific concentration of lactic acid, the retina has to produce more lactic acid than to fill a small eye with the same concentration of lactic acid. The total lactic acid per eye is a good indicator of the glycolysis rate in the retina. We have presented the lactic acid concentration in aqueous humor and vitreous of Black Moor and common goldfish.[32] The lactic acid concentration increases as the Black Moor goldfish increase in size and eye volume. On the contrary, the lactic acid concentration decreases as the common goldfish increase in body size with a constant eye volume.

Since anaerobic glycolysis in the retina is higher than that in any other tissues, the high lactic acid in the vitreous must be related to an active metabolism in the retina of a growing eye. The glycolysis rate is dependent on the activity of phosphorylase and hexokinase, the enzymes that initiate glucose

utilization. Future study should be directed to these enzymes and their possible role in the regulation of eye growth.

Peptides in vitreous humor

The present data revealed a dramatic difference in the vitreous peptides between small and large eyes. The chromatographic method used in this study separates peptides according to their hydrophobicity.

The major known macromolecules in the vitreous are hyaluronic acid, collagens, and glycoproteins with molecular sizes exceeding 200,000. They are heterogeneous in size. Peaks 2 to 4 could be the major macromolecules of fish vitreous. These peaks contain heterogeneous mixtures of peptides that cannot be characterized by mass spectroscopy. The remarkably high amounts of highly hydrophobic small peptides (**Figure 11**, peaks 6 to 10) in fish are interesting new findings. Future investigations into the amino acid composition and sequence of these peptides will help to understand the significance of the altered protein synthesis in the vitreous cavity that supports the unrestricted expansion of eye size among Black Moor goldfish.

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