FISKERIDIREKTORATETS KJEMISK-TEKNISKE
FORSKNINGSINSTITUTT

PROTEINS IN FISH MUSCLE

BY

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Contents:

Abstract.

(1) The proteins of muscle fibrilles, tropomyosin, actin, myosin and actomyosin. 65 % of fish muscle protein. (40 % in mammals).

(2) The proteins of cytoplasma (enzymes in muscle metabolism), globulin x, myogen, myoglobin: 26-30 % of fish muscle protein (35-40 % in mammals).

(3) The proteins of the connective tissue, stromaproteins (collagen), 3 % of the muscle of teleost. 10 % in elasmobranch. 17 % in mammals.

Group 1, extractable with neutral salt solution of high ionic strength - 0,5.

Group 2, extractable with neutral salt solution of low ionic strength - 0,15.

Group 3, not extractable with neutral salt solution, nor in diluted acids and alkalies.

Group 1 has great crystall lattice energy and great dipole moment; this results in great sensitivity towards salt ions.

Group 2 is rich in charged side chains, but has a small dipole moment; this results in a lower sensitivity towards salt ions.

Precipitation of fish proteins: % saturation of ammonium sulphate.

Tropomyosin	50 - 66
Myosin	35 - 48
Actin	10 - 20
Actomyosin	28 - 38

Result of electrophoresis:

Both pure actomyosin and pure actin give one sharp peak. Pure myosin gives a broader peak; this may indicate some heterogeneity. Myogen gives 7 components by Tiselius-electrophoresis. Each species of fishes gives a different pattern. Electrophoresis in starchgel gives 12 components at pH 7,5.

Results by ultracentrifuge:

Both actomyosin and tropomyosin give 1 sharp peak. Myosin is not homogeneous. Myogen gives 4 components.

Crystallized fish protein:

Myogen proteins, myoglobin and tropomyosin.

In the white muscle, glycogenic metabolism predominates; in the red muscle (Musculus lateralis superficialis Trunci), the citric acid cycle probably prevails.

The red muscle (e.g. in tuna) seems to be a passive muscle with a high oxygen metabolism. The white muscle is an active muscle with low oxygen supply.

Introduction.

The muscles consist of fibre cells, in which lie bundles of chain-molecules, and round the fibrilles is a clear solution, which we call sarcoplasma. During the development of the muscle, the nucleus of the cells is displaced, so that it comes close to the cell walls.

The fibres and bundles of fibres are surrounded by a number of membranes. (1)

Along the lateral line of the fish, there is a red superficial muscular region underneath the skin, called the red muscle or Musculus lateralis superficialis Trunci, which differs in constitution from the rest of the muscle tissue. (2, 3).

The muscles in the fish correspond to nearly 50 % of the total weight. The general composition of the fish muscle (in per cent of weight: (4, 5) Protein and nitrogen extraction (N·6,25) - 16,3 - 19 (of this total, pure proteins make 14 - 18 %).

Water	75 - 83 %
Lipids	0,2 - 20 "
Ash	0,2 - 1,4 "

The proteins of muscle may be classified in: (1) The proteins which give the muscle its power of contraction. These are tropomyosin, actin, myosin and actomyosin, a complex of actin and myosin. These proteins represent ab. 65 % of the muscle proteins of fishes, against only 40 % of these of mammals. They are extractable with a salt solution of high ionic strength (- 0,5).

(2) The proteins which constitute the enzymes of the muscle metabolism. There are two groups: myogen which is found soluted in the sarcoplasma, and globulin x which seems to come chiefly from the mitochondria. Both are extractable at low ionic strength - 0,15. But according to its name, globulin x is insoluble in water when the conductive power decreases to 10^{-4}, whereas myogen is an albumin and soluble in water.

They compose 26-30 % of the protein in fish muscle, in nearly equal amounts. In mammals there are 35-40 %.

(3) The stroma proteins, which form the connective tissue of the muscle. They consist of the glueforming collagen (6), which is not soluble in neutral salt solution, nor in diluted acids and alkalies. They amount only to 3 % in teleost muscle, 10 % in elasmobranch muscle, against 17 % in mammals (7).

Preparation of the fish proteins.

The actomyosin is prepared by extracting a muscle pulp by means of a bicarbonate-buffered 0,5 mol potassium-chloride solution, pH 7,5 at 0°C. The remnants are spinned down, and the actomyosin is precipitated by diluting it to a ionic strength below 0,35, and the precipitate is washed with water. Then it is soluted in a salt solution. One repeats the whole process several times to get a clean actomyosin solution.

Clean myosin is particularly intricate to make from muscle of fish.

One proceeds nearly as with actomyosin, but to the extraction solution one adds adenosine-triphosphate, shortened ATP, to keep actomyosin dissociated in actin and myosin, and versen (also called titriplex), to bind earth alkali ions. In this way, the ATP-ase effect of myosin is inhibited.

One removes the actomyosin from the myosin solution by dialysing it against a buffer solution with ionic strength 0,25 and pH 7,1. So the actomyosin falls out. (28, 33).

The method of isolating actin is so surprising that one must admire the men who developed it. (9, 10). One washes the grinded fish fillets some times with absolute alcohol and then several times with ether. From the dried fibre mass one extracts the actin with a sodium-chloride solution pH 7,3 and ionic strength = 1,0 (26) or with water (78).

This actin is still contaminated with tropomyosin, but by centrifuging for 2 hours with 40,000 rotations a minute, one gets 3 layers in the centrifuge glass, the layer on the bottom being pure actin. (11, 26).

The proteins of sarcoplasma are obtained by extractin the minced fish muscle with cold water. By dialysing the solution against water for 64 hours, the globulin x group is precipitated, and is no longer able to be disolved again.

This denaturation is characteristic for this group and makes it difficult to investigate.

The myogen group contains nearly 50 enzymes. By cautious addition of $(NH_4)_2SO_4$ with a little sodium-phosphate, or with alcohol, one succeeds in dividing the myogen group into a series of fractions with different degrees of solubility. These fractions are further separated by electrophoresis, ultracentrifugation and crystallisation.

Together with the myogen proteins is the red protein, myoglobin. This can be purified by 0,5 m basic lead acetate at 30°C and precipitated at 80-95 % saturation of ammonium sulphate. (12, 13, 14).

As the electrophoretic pattern shows, the myoglobin represents the major part of the red pigment of the red and the bright muscle in tuna (Thynus Thynes). It forms one single peak in tuna and two peaks in carp. (12). Its contents can be determined by the alcaline haematine method. The amount of myoglobin in the bright muscle varies from 0,02 to 0,08 units of Klegg and King hemoglobin standard, in the dark muscle about 0,14. There is no relation between the myoglobin contents and the size of the tuna. (15, 16).

Physical and chemical properties of fish proteins.

At their iso-electric point (pH 4-5,5) where the proteins are electrically neutral, they are least soluble and they can easily denaturate.

By the addition of a neutral salt-like sodiumchloride, the isolectric point will be displaced to a lower pH.

In the alcaline region the addition of salt will increase the solubility of the proteins. This particularly concerns the myosin-group, which is made of proteins with great crystal lattice energy and a great dipole moment.

The myogen group, on the contrary, belongs to the water-soluble proteins, rich in charged groups, but with a small dipole moment. (17).

The extractibility of muscle proteins of cod increases from 13 % at pH 5 to 95 % at pH 6, with a salt solution of ionic strength 0,85. (2, 18).

The ability to swell and increase in hydration in the alcaline region can be explained by an increase in acid groups, particularly carboxyl groups, or by reduction in the number of basic groups in the protein.

Calcium-ions reduce swelling. It may be explained by calcium-ions blockading the carboxyl-groups, thus reducing the number of acid groups.

If one adds sodium-chloride to an acid fish protein solution, the salt has the opposite effect; it reduces the swelling and solubility of the proteins.

The salts which have the greatest swelling and solubilizing effect are generally those with multivalent cations. But this is influenced by several factors, so one has to be cautions with this simplification of facts.

If one increases the salt concentration beyond a certain limit, the proteins are precipitated.

The farther away we are from the isoelectric point, the higher is the salt concentration which is needed. This effect is explained by the salt ions absorbing the polar molecules of the solution and displacing the protein molecules, so that these precipitate.

The salts with the highest precipitating effect are those with multivalent an-ions, but in many salts the saturation occurs at so low a concentration that precipitation is not possible.

The per cent saturation of ammoniumsulphate for precipitation is for:

Myoglobin ab. pH 7	(tuna)	75 - 95 % (2)
Myosin		35 - 48 " (19, 2)
Tropomyosin at pH 5,2	(carp)	50 - 66 " (2)
Actomyosin		28 - 38 " (18)
Actin		10 - 20 " (18)

Generally an increase of temperature enlarges the solubility of proteins in diluted solutions and enlarges the precipitation of proteins in more concentrated salt solutions.

The mobility of fish proteins are closely connected to what is said about the isoelectric point and charged groups.

To get a picture of the electrophoretic behaviour of the fish proteins, diagrams of the fish proteins at the range of pH 4 to 9 have been made. (20).

The electrophoresis of fish proteins, extracted at high ionic strength is difficult, particularly for smaller components, because of the viscosity of the solution.

At pH 7,4 one gets a sharp peak of actomyosin.

Myosin gives a broader peak of a little slower velocity. (21).

It has been shown that it aggregates more easily than rabbit myosin. (78).

The myogen and globulin x group both show a rather corresponding electrophoretic velocity, and higher than rabbit. (19, 22).

The electrophoretic pattern of each species of fishes differs so much that is may be used as a sort of "finger-print" for the species. (23, 24).

By means of electrophoresis in starchgel at pH 7,4 one has obtained better separation of the myogens. One distinguishes 12 gradients by this method, against 7 by means of electrophoresis in buffer solution or on paper. But this method is troublesome, and it is difficult to measure the area of the peaks exactly. (25)

By electrophoresis the proteins are separated and characterized, but as the composition of the amino acids in the enzymes seem to change from one species of fishes to another, the method needs to be supplied with enzymologic measurements to reveal the laws and relationship of the species of fishes.

Ultracentrifugal studies.

By means of ultracentrifuge one has measured the sedimentation of fish proteins. One gets an idea of the molweight and the structure, and it also assists in the control of the purity of protein preparates.

It has been difficult to obtain a fish myosin with one sharp peak. (21, 26). This may have the same reason as mentioned for electrophoresis.

The ultracentrifuge diagram of the great myogen group gives only 3-4 peaks, but in combination with fractionation with ammoniumsulphate and following electrophoresis, one finds that generally the myogen fractions precipitating by increasing salt saturation tend to increase in electrophoretic mobility and to decrease in sedimention velocities; but there are several exceptions. (22).

Viscosity.

The measurements of viscosity by means of an Ostwald viscosimeter has been used to follow the formation of actomyosin from actin and myosin, and the study the dissociation of actomyosin by addition of adenosine-triphosphate.

The increase in viscosity by formation of actomyosin seems higher in fish than in mammals, and because the number of free sulphydrylgroups in myosin is responsible for the increase of viscosity, this gives a slight indication that myosin of fish has more free sulfhydryl groups than in mammals. Unfortunately, the values vary in the various preparations. (2, 26, 27).

The intrinsic viscosity of fish proteins:

Actomyosin	5,0 - 5,4	(in rabbit 3 - 5)
Myosin	2,0	(in rabbit 2,2)
G-actin	0,075	(in rabbit 0,20)

The measurements of viscosity are limited to a short range of concentration near 0,2 % actomyosin. At too high a concentration the thixotropy interferes, at too low a concentration one gets disturbances by agglutination. (28).

Crystallization.

It has been possible to crystallize several myogen proteins from carp, plaice and cod after fractional precipitation with $(NH_4)_2SO_4$ or with alcohol (25), further to crystallize myoglobin from tuna and carp, and besides nucleotropomyosin. The proteins have proved to be homogeneous by electrophoresis and ultracentrifugation. (2, 29, 22).

Spectrophotometric examination.

In oxy-myolobin of tuna three maxima are found in the visible region, one at about 415 mμ, and two smaller ones at 540 and 575. This corresponds to that of mammals. (2, 9).

The ultraviolet absorption of carp nucleo-tropomyosin has shown 10 to 20 % ribonucleic acid. (2). The ultraviolet spectrum of a myogen fraction of plaice and carp which was precipitated at 90-100 % $(NH_4)_2SO_4$ showed that this protein contained no tyrosin and tryptophan, but nearly 17 % phenylalanine. (22, 29).

Influence of fatty acids on proteins.

It has been observed that the fatty acids not only influence juiciness, tenderness and aroma, but the fatty acid anions also stabilize the protein structure of fish muscle, prevent the breaking of hydrogen bridges, and make the proteins more stable against heat as well as against freeze denaturation. The maximal effect is considered to be obtained by 8 moles fatty acids with 12 carbon atoms per cent mol protein. (30, 31, 32).

Metabolism of the muscle.

In the relaxed muscle, myosin forms a stable complex with adenosine-triphosphate (ATP) and potassium, calcium and magnesium ions.

By the contraction of the muscle, myosin, by means of its ATP-ase property, splits off a phosphate group from ATP, and the great energy which is set free is used by myosin to form a contracted actomyosin compound.

There are in the muscle 2 systems producing ATP, the first going out from lipids, the citric acid cycle, the second is the glycolytic system.

In the original system, which we found in the smooth muscle and in undifferentiated muscles of foetus, the enzymes of the citric acid cycle dominate compared to glycolysis. (34)..

At present this phenomenon is being studied to see if this to some extent is the case with the red muscle with its low muscle activity.

In the skeletal muscle like the ordinary white muscle of fish, there are nearly 10 times more glycolytic enzymes than of the enzymes of the citric acid cycle. (34). Ordinarily the circulation of blood in a passive muscle is low (35), and consequently there is a low metabolism. This one should expect with the red muscle.

However, the red muscle is rich in blood capillaries (36, 37). It has a high content of myoglobin and probably of the enzymes of the cytochromic system, properties, distinguishing an active tissue. (38, 39, 40). Notable is also the fact that it lies parallel to the white muscle with its low supply of oxygen (2).

It is tempting to say that there must be some sort of metabolic co-operation between the red and the white muscle. (3).

Time will show if such a differentiated partition of functions, which is exceptional in physiology, is indeed possible.

Changes in protein structure during preparation of fish products.

When a fish dies and the circulation of the blood stops, the muscle glycogen disappears, by decomposition to lactic acid. Then follows that no more adenosine triphosphate (ATP) is rebuilt to replace the ATP which is used as energy source for muscle activity. (41, 42, 43).

Myosin and actin now form an ATP-free and extremely rigid form, which gives the muscle its property at rigor mortis.

The rigor disappears by bacteriological breakdown of the tissue. (41).

A high contents of glycogen in the fish muscle result in a longer pre-rigor period of storage, and produce a higher acidity in the muscle. The latter causes a lower bacterial growth and

activates the proteolytic enzymes and thus develops a more tasteful and tender product. (44).

To obtain a high glycogen contents it is important to catch and kill the fish instantly without struggling. The advantages of electric catching or killing are obvious. (46, 47, 48).

It is a question if it has any advantages to use pre-rigor fish for fish products. For the frozen fish industry this is not clear, but probably this is of no great importance. (49).

In 2 % brine-solution, pre-rigor fish has been reported to take up nearly 25 % more water during 26 hrs, than fish in rigor. (45).

In the living muscle myosin binds ATP and also a relatively great amount of potassium-ions. At few hours after death, myosin loses this capacity. As at the same time there occurs a reduction in the water-binding capacity, (50), there may be a connection between these things. (51).

At products in which proteins with maximal water-binding properties are important, pre-rigor fish are to be used. (50).

There are varying opinions about the importance of the stroma proteins on the properties of fish products, but if one considers that the permeability of the membranes influence the velocity by which solutions and salts diffuse through the tissue, and the velocity with which the enzymes and microorganisms penetrate into the muscle (52, 53), further that the precence of a higher content of stroma proteins should stabilize the internal structure of the muscle cell, then it seems natural that differences and changes in the stroma proteins influence the condition of fish after storage

The velocity by which salts penetrate membranes, decreases in the following sequence: potassium, sodium, calcium and magnesium, and for the anions from chlorions to sulphate-ions.

By means of hyaluronidase it is possible to increase the permeability. (54).

The flavor of fish accompanies mainly the juice of the fillet. To avoid loss of flavor, one has to prevent loss of juice during cooking and leaching of the fillets. (55, 56).

By freezing of the fish there is an increase of salt in the waterphase and this will increase the solubility of the proteins and may loosen the protein structure. (50, 57, 58). But the formation of ice crystals highly reduces the water phase. Now the molecules which are rich in charged groups and with a great dipole moment, such as myosin, will be precipitated, and then slowly become insoluble. (62, 63).

After 12 days at -20°C, around 1/3 of the myosin was denatured, but the myogen was undamaged. (64). Isolated, freeze dried myogen however, seems sensitive to denaturation. (25).

The curves for freeze denaturation differ much, both for the different fishes and species of fishes, and with alternating contents of lactic acid and fat, and with the various authors. (43, 65, 66, 67, 73, 77, 59). By extraction with unbuffered salt solution, the amount of protein from cod muscle decreases rapidly during the first 12 days. With bicarbonate buffered salt solution, there is a more even sinking during some months. (25).

As the potassium ions influence the osmotic pressure of the cells (50, 68) the loss of potassium which follows the drip may contribute to give frozen fish its tough consistency.

From 100 g of muscle which contained 370 mg potassium, 24 ml serum with around 1/3 of the potassium contents of the muscle was pressed out after freezing and thawing. During storage in 10 months at -20°C, the presswater increased from 24 to 40 ml. (26).

It has been tried to reduce the amount of drip by addition of polyphosphate. This is partly obtained by keeping a higher pH in the muscle. This must be done at the expense of the advantage of a low pH and of the loss of time in soaking the muscle with ½ % polyphosphate. Polyphosphate-treated fish muscle gives a slightly decreased drip and a satisfactory taste, and it partially conserves the ATP in the muscle by blockading the ATP-ase enzymes. (69).

During drying of fish for production of fish meal, the proteins denaturates at 60-70°C, with the exception of tropomyosin, which is surprisingly stable up to nearly 120°C. By passing 100°C, the proteins often in combination with carbohydrates and lipids may be transformed into compounds of lower nutritive value. The papers in this field show remarkable divergence (70, 71, 72, 73, 74), however, by careful production of fish meal none of the ordinary methods seems to reduce the nutritive value of the proteins particularly. (69, 75, 76).

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