Pink lateral muscle in the carp (*Cyprinus carpio* L.): histochemical properties and myosin composition[1]

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Summary. The pink muscle of the carp differs from the white (and red) muscle not only histochemically but also in its myosin isoform, as shown by peptide maps of the myosin heavy chains. Results of an electrophoretic analysis of myosins are discussed in the light of their immunohistochemical properties and histochemical ATPase activity.
Key words. Fish; pink muscle; myosin; (immuno)-histochemistry.

In most teleosts, the superficial red and deep white layers of the lateral muscle are separated by an intermediate layer, often called pink because of its colour. Little is known about the function or morphological profile of the pink muscle layer, which, although it is usually small in relation to the total amount of lateral muscle, shows species-related variations in size and histochemical and immunohistochemical properties, being always clearly distinguishable from the red muscle, but showing some similarities to the white muscle[2-8]. In the past, the pink muscle was often viewed as a transition zone containing fibres able to develop into the red or white types[5,9], but recent work has pointed to a morpho-functional specialisation[10,11]. In this context one of the best-studied species is the carp (*Cyprinus carpio* L.), which has a well-developed pink muscle layer. However, as the histo- and immunohistochemical differences between fibres of the pink and white mosaic muscle[8,12] were not accompanied by any differences in myosins, as shown by one-dimensional electrophoresis[10], we decided to examine this species in more detail, using the more sensitive two-dimensional electrophoresis and peptide mapping of myosin heavy chains in addition to (immuno)histochemical methods.

Cryostat sections of carp lateral muscle were stained as follows: a) histochemically for Ca-dependent myosin ATPase activity (mATPase) (ref. 13, method A), modified as described in [14] and for Ca, Mg-dependent actomyosin ATPase activity (amATPase)[15] but increasing the incubation time to 45–60 min and,

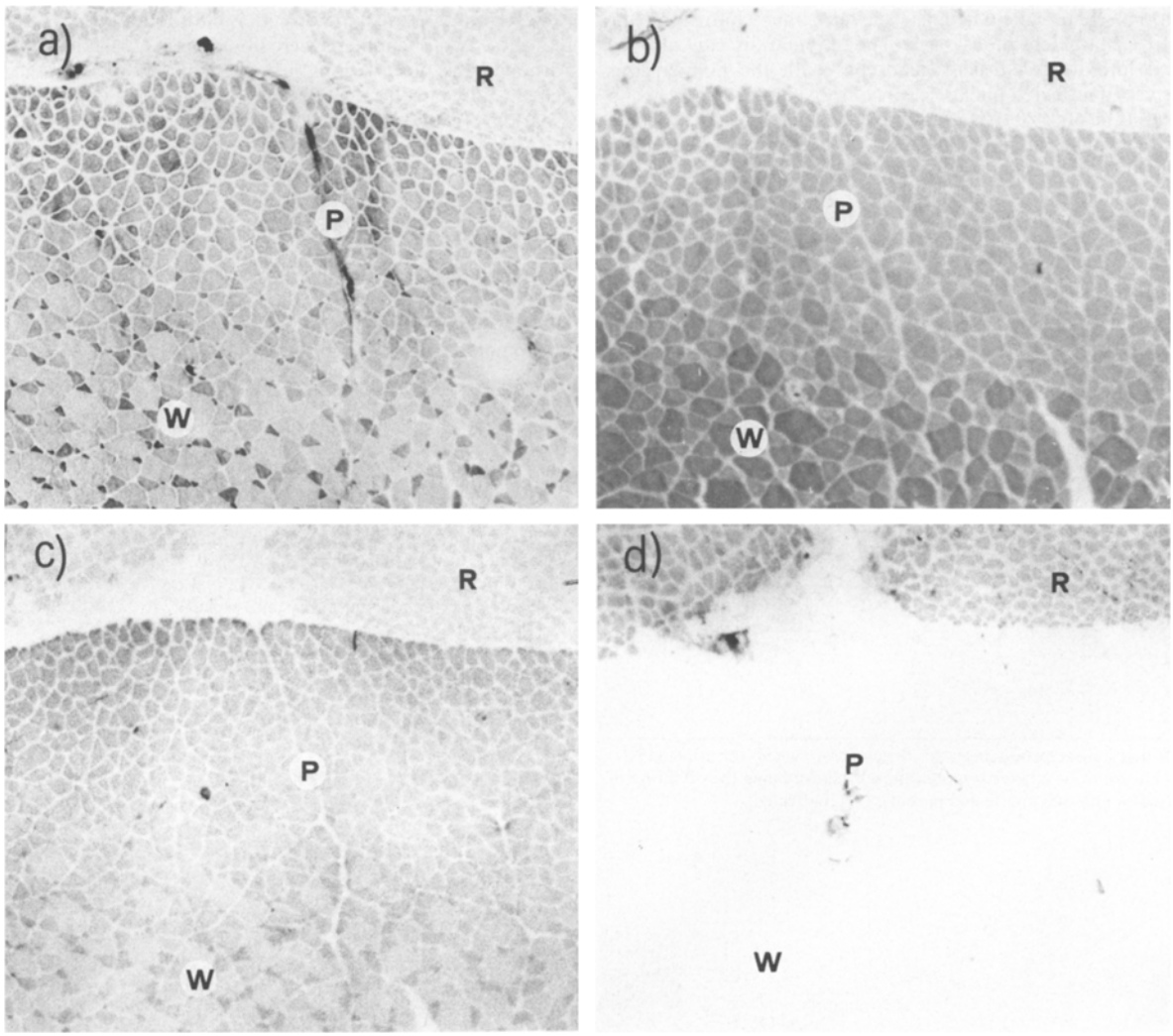

Figure 1. Transverse sections of the lateral muscle of the carp: red (R), pink (P) and white (W) portions. *a* mATPase, pH 4.7 preincubation; *b* amATPase; *c, d* indirect immunoperoxidase staining with anti-F (c) and anti-SHC sera (d). × 50.

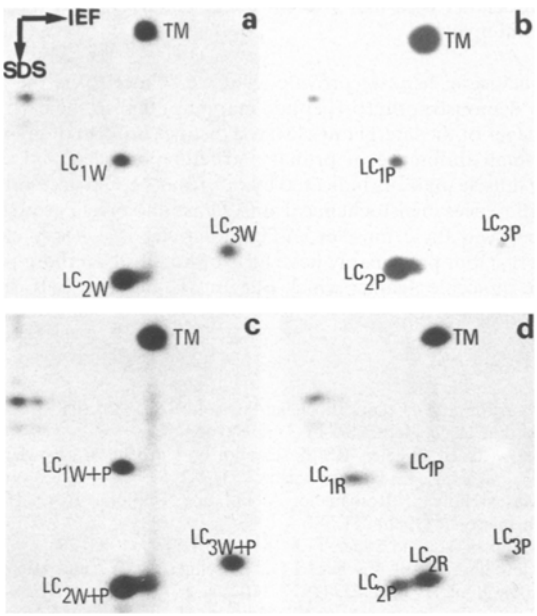

Figure 2. 2-D gels of ^{14}C-labelled myofibrillar extracts of: *a* white (W); *b* pink (P); *c* co-migration of W+P and *d* co-migration of P+Red (R) muscles of carp. Proteins were visualized by fluorography (2 weeks exposure). Only the part of the gels containing the light chains (LC) and tropomyosin (TM) is shown.

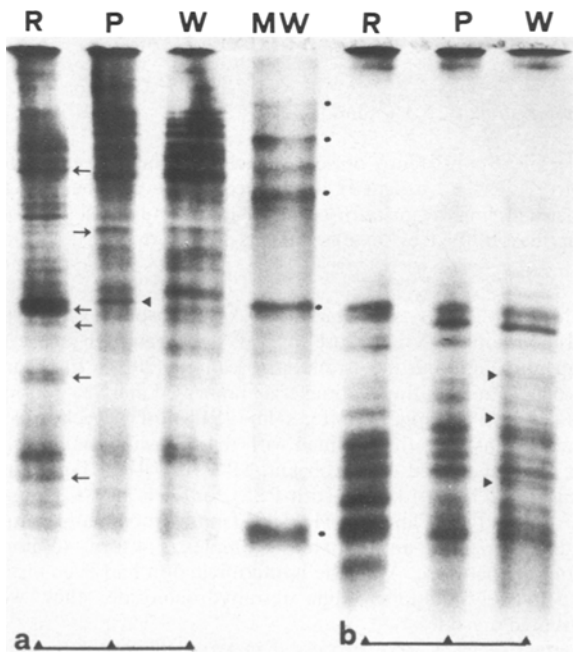

Figure 3. 1-D peptide mapping of ^{14}C-labelled myosin heavy chains from red (R), pink (P) and white (W) lateral muscle of the carp. Proteins were visualized by fluorography (5 weeks exposure). Maps in *a* were obtained by digestion with 10 µl chymotrypsinogen at 0.2 mg/ml and those in *b* with 10 µl *Staphylococcus aureus* V_8 at 0.01 mg/ml. The major differences between red and pink are indicated by arrows, and those between pink and white by arrowheads. MW: molecular weight markers (spots), kDa 92.5; 66.2; 45; 31; 14.

b) immunohistochemically by the indirect immunoperoxidase method, using antibodies raised against myosins from the lateral muscle of *Mugil capito,* and specific for whole red (anti-S) or white (anti-F) muscle myosins, and for the heavy chains only of these myosins (anti-SHC and anti-FHC respectively)[8].

Samples of myofibrillar preparations obtained from histochemically identified areas of carp red, pink and white muscle, and ^{14}C-labelled single red, pink and large white fibres from the same regions, were analysed by 1- and 2-dimensional SDS polyacrylamide gel electrophoresis[8], and by peptide mapping of heavy chains[16] (method modified in the case of single fibres as in Billeter et al.[17]).

Histochemical mATPase activity was equally high in the pink and white muscle except after acid (pH 4.7–4.3) pre-treatment which results in stronger staining of many and small white fibres because of their greater acid-stability compared to the large white fibres (fig. 1a). AmATPase activity, however, was found to be very low in red muscle, high in white muscle, and intermediate in all pink muscle fibres (diameter range 10–70 µm) (fig. 1b). Since the contraction speed of muscle fibres is related to their actin-activated myosin ATPase activity, the histochemical amATPase result suggests that the pink muscle fibres have a contraction speed intermediate between that of the red and the white muscle. This is consistent with the recruitment order during swimming at increasing speeds: first red, then pink then white muscle[10].

Immunohistochemically, pink muscle is easily distinguished from red muscle, but not from the white muscle, and like the latter gives a positive reaction with anti-F and anti-FHC but no reaction with anti-S or anti-SHC (figs 1c, 1d). This result is similar to that of Akster[12], who found that antibodies specific for carp red and white myosins failed to distinguish between pink and white fibres, both of which reacted with anti-white but not anti-red sera. The reaction with our anti-F is however slightly stronger in the pink and small white fibres than in the large white and the small fibres of the white muscle cross-react weakly with anti-S.

Analysis of myosin light chains by 2-dimensional gel electrophoresis showed that the 3 light chains of pink muscle myosin (LC_P) are indistinguishable in apparent molecular weight and isoelectric point values from those of white muscle myosin (LC_W). In co-migration experiments in which samples of pink and white muscle myofibrils are combined and run on the same 2-dimensional gel (fig. 2 c) the pink and white muscle myosin light chains migrate to the same positions, and only these 3 light chain spots are found even on long-exposure fluorograms of ^{14}C-labelled material. In contrast, the light chains of red muscle myosin (LC_R) are different in both apparent molecular weight and isoelectric point (fig. 2d).

Myosin heavy chains from the same myofibrillar samples for analysis of light chains were examined by 1-dimensional peptide mapping. The same conditions of digestion of the heavy chains and electrophoretic separation of the resulting peptide fragments apply to all samples, and thus differences in peptide patterns (maps) should be due only to differences in the primary structure of the heavy chains. As might be expected, the difference in heavy chain peptide maps between red and white muscle myosins is greater than that between pink and white muscle myosins. However, although the pink and white muscle myosins give similar maps they are not identical, and there are some clear differences between them (fig. 3).

Myofibrillar preparations of white muscle myosin will of course also contain, in addition to the predominant isoform in the large white fibres, a minor component derived from the small white fibres. Could the pink muscle myosin be the same as that in the histochemically similar small white fibres? We think not: some of the bands specific for pink muscle myosin in the HC peptide maps are quite strong, yet do not appear even in trace amounts in the white muscle myosin maps, and furthermore, pink and small white fibres also have slightly different immunoreactivities

with our anti-S antibodies. 2-Dimensional gels of myosin light chains, and peptide maps of myosin heavy chains from ^{14}C-labelled single fibres of red, pink and white muscle gave results identical to their equivalents obtained from myofibrillar preparations.

Thus, the light chains of pink and white muscle myosins from *Cyprinus carpio* are indistinguishable not only in apparent molecular weight[10] but also in isoelectric point. The heavy chains of these myosins, however, give slightly different peptide maps, suggesting that there are small differences in their amino acid sequences. An analogous situation, i.e. the association of light chains of the same type with different heavy chains, is found in mammalian skeletal muscle, in which the same 'fast' light chains are associated with the different heavy chains of IIA, IIB and neonatal myosins[17,19].

In conclusion, here we provide what we believe to be the first direct demonstration (by peptide mapping) that in the carp the pink layer of the lateral muscle has a distinct isoform of myosin. The small difference in primary structure between pink and white muscle myosins indicated by our results could account for the differences in histochemical amATPase and mATPase activities between these muscles (ATPase activity is a heavy chain property), but presumably have little or no effect on those parts of the molecule against which our anti-F and anti-FHC antibodies are directed.

1 Work supported by the M.P.I. (40%).
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0014-4754/87/040384-03$1.50 + 0.20/0

Substance P-like immunoreactive fibers in the frog taste organs

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Summary. In the frog tongue, substance P(SP)-like immunoreactive fibers were consistently present in each fungiform papilla, which contained the gustatory apparatus. Numerous SP-like immunoreactive fibers were usually distributed in the periphery of the gustatory disc and formed a varicose meshwork among the ciliated cells encircling the gustatory disc. SP-like immunoreactive fibers were rarely evident inside the gustatory disc. The role of SP-containing fibers in the frog taste organ was discussed.
Key words. Substance P; immunohistochemistry; taste organs; frogs.

Recent immunohistochemical studies demonstrated the presence of substance P(SP)-containing fibers in the taste buds in a few species of mammals[1-3], findings which suggested that SP may be involved in gustatory processes[1,3], as well as in other sensory processes, particularly those related to pain transmission[4]. However, the exact role of peripherally transported SP in the taste organ remains to be elucidated. Frog taste organs have structures different from those in other species[6] and are frequently used in physiology-related studies. There has apparently been no report on the presence of SP in the frog taste organs. In order to clarify the functional role of SP in the taste organ, it is important to elucidate the anatomical association between the SP-containing fibers and the taste apparatus in the frog tongue. The present study is concerned with the immunohistochemical demonstration of SP-containing fibers in the fungiform papillae of the frog tongue.

Materials and methods. The tongues of frogs (*Rana catesbiana*, weighing 240–400 g) were investigated.

Immunohistochemical procedures. The frogs were anesthetized with 5% urethane, and perfused with a cold solution of 0.2% picric acid and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Small blocks of the tongues were immersed in the same fixative for 2 days, then placed in 0.1 M phosphate buffer (pH 7.4) containing 15% sucrose for 2 days, frozen with dry-ice in isopentane and cut into transverse sections (20 μm thick) in a cryostat. Free floating sections were immersed in 0.3% Triton X dissolved in phosphate buffer saline (PBS) for 1 week at 4°C. The sections were then stained with the following materials, in the order indicated: 1) rabbit anti-SP serum (IBL, Japan) at a dilution of 1:200 (diluted with PBS) for 24 h at 4°C; 2) goat anti-rabbit IgG (Cappel) (1:40) for 40 min at room temperature; and 3) peroxidase-antiperoxidase complex (1:40) for 40 min at room temperature. After the immunoreaction had been visualized with diaminobenzidine tetrahydrochloride, they were mounted on slides.

Control sections were processed in parallel, and in the same manner, except that they were incubated with primary antiserum preabsorbed (0.063 mg/ml) with synthetic SP (Peptide Institute Inc., Japan) or only PBS instead of primary antiserum.

For the immunoelectron-microscopic study, the method developed by Somogyi and Takagi[5] was used. In brief, the frogs were anesthetized with urethane and perfused with picric acid (0.2%)-paraformaldehyde(2%)-glutaraldehyde (0.15%) fixative. Small blocks of the tongues were immersed in picric acid (0.2%)-para-