



Melanocortin systems on pigment dispersion in fish chromatophores

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α -Melanocyte-stimulating hormone (α -MSH) is responsible for pigment dispersion in the chromatophores of fish and other tetrapods such as amphibians and reptiles. Recently, we discovered that α -MSH did not always stimulate pigment dispersion because this hormonal peptide exerted no effects on the melanophores of flounders. We assumed that the reduction of α -MSH activity was related to the co-expression of different α -MSH receptor subtypes – termed melanocortin receptors (MCR) – a member of G-protein-coupled receptors (GPCR) – based on several reports demonstrating that GPCR forms heterodimers with various properties that are distinct from those of the corresponding monomers. In this review, we summarize the relationships between the pigment-dispersing activity of α -MSH-related peptides, molecular forms of α -MSH-related peptides, and *mcr* subtypes expressed in fish chromatophores.

Keywords: melanocyte-stimulating hormone, melanocortin, melanocortin receptor, G-protein-coupled receptor, heterodimer, pigment-dispersing activity

INTRODUCTION

Fish color change is achieved by two major aspects, such as physiological and morphological color change. Short-term physiological color change in teleosts is caused by pigment aggregation/dispersion in skin chromatophores in which the neuroendocrine and sympathetic nervous systems are involved (Fujii and Oshima, 1986; Fujii, 2000). In the long term, these systems also influence survival or apoptosis of the chromatophores and contribute to morphological color change (Sugimoto, 2002).

The hypothalamo-pituitary system is a major player of the neuroendocrine system, in which melanocyte-stimulating hormone (MSH) and melanin-concentrating hormone (MCH) are associated with color changes in fish, exerting opposing actions (Takahashi and Kawauchi, 2006a; Mizusawa et al., 2011). MSH stimulates pigment dispersion and MCH induces pigment aggregation.

Melanocyte-stimulating hormone is derived from a precursor termed proopiomelanocortin (POMC), and MCH is derived from a precursor called proMCH (Takahashi and Kawauchi, 2006a). The major sources of MSH and MCH are the pituitary gland and hypothalamus, respectively. The MSH receptor, known as the melanocortin (MC) receptor (MCR), is a member of the G-protein-coupled receptor (GPCR) family, as is the MCH receptor. The members of this family have seven transmembrane domains

(Mountjoy et al., 1992; Saito et al., 1999). Five subtypes and two subtypes are present for MCR and MCH receptor, respectively (Takahashi and Kawauchi, 2006b).

Compared to the rather conservative amino acid sequence of MCH, MSH varies in molecular form because of the presence of two or more molecular forms encoded in *pomc* and a variety of modifications to the N- and C-terminals (Ebelre, 1988). Recently, we discovered that the biological activities of α -MSH-related peptides were dramatically reduced by the presence of an acetyl group, and this modulation was related to the co-expression of different *mcr* types (Kobayashi et al., 2009, 2010). Abundant evidence regarding this interesting activity modulation was obtained in an experiment using barfin (bf) flounder, a flatfish that is commercially important in the northern part of Japan. Comparative studies using the closely related Japanese (Jpn) flounder (Kobayashi et al., 2012) and the distantly related goldfish (Kobayashi et al., 2011) further supported these results. In this review, we provide an overview of our recent studies on the relationships between *mcr* transcripts expressed in fish chromatophores and the biological activities of α -MSH-related peptides possessing different numbers of acetyl groups. The differences in activity of these peptides appear to be related to the interaction of ligands with different degrees of acetylation at the N-terminus and the types of MCR biosynthesized in the chromatophores.

GENERAL ASPECTS OF MC SYSTEMS AND CHALLENGES PROVIDED BY α -MSH

Melanocortins are members of peptides such as α -MSH, β -MSH, γ -MSH, and adrenocorticotrophic hormone (ACTH). These peptides are derived from a common precursor protein termed proopiomelanocortin (Smith and Funder, 1988; Castro and Morrison, 1997; Raffin-Sanson et al., 2003; Kasper et al., 2006; Takahashi and Kawauchi, 2006b). POMC also contains β -endorphin (β -END), a non-MC peptide. In general, α -MSH is composed of 13 amino acid residues with an acetylated N-terminal and an amidated C-terminal. α -MSH is derived from the N-terminal portion of ACTH comprising 39 amino acid residues, and other MSHs originate from different parts of POMC. While the POMCs of basal ray-finned fish, lobe-finned fish, and tetrapods contain three MSHs, those derived from ray-finned fish, namely teleosts, lack γ -MSH, but contain α -MSH and β -MSH (Takahashi and Kawauchi, 2006a). Sharks and rays are unique because of the presence of four MSHs; the fourth MSH is termed δ -MSH (Amemiya et al., 1999, 2000; Takahashi et al., 2004, 2008). γ -MSH appears to have been deleted from POMC and δ -MSH is suggested to be derived from β -MSH during the evolution of teleost and cartilaginous fish, respectively (Takahashi and Kawauchi, 2006a).

A major source of POMC is the pars intermedia (neurointermediate lobe, NIL, in teleosts) and pars distalis (PD) of the pituitary gland, and the final products in each lobe are different because of tissue-specific post-translational processing (Smith and Funder, 1988; Castro and Morrison, 1997; Raffin-Sanson et al., 2003; Kasper et al., 2006; Takahashi and Kawauchi, 2006b). For example, ACTH is a product of the PD, and α -MSH is a product of the NIL.

The presence of five subtypes – MC1R to MC5R – has been reported for MCR (Mountjoy et al., 1992; Mountjoy, 2000; Gantz and Fong, 2003). These subtypes are distributed in bodies in a rather subtype-specific manner, and are associated with a variety of biological processes conveyed by MC peptides. Of the subtypes, MC1R and MC2R are the classical α -MSH and ACTH receptors, respectively (Mountjoy, 2000; Gantz and Fong, 2003). Many experiments on the binding affinity of each subtype vs. a variety of MC peptides have been conducted using a solely expressed MCR molecule in a transfected cell line (Cone, 2000; Schiöth et al., 2005). These procedures have characterized a remarkable difference between MC2R and the other MCRs – while MC2R binds ACTH, but not MSH, the other MCRs bind both ACTH and MSH. These methods have also contributed to the development of many MC peptide-related antagonists and agonists (Cone, 2003). However, increasing biochemical and biophysical evidences have indicated that GPCRs have been shown to form heterodimers or heterooligomers with various biochemical and/or pharmacological activities that are distinct from those of the corresponding monomers or homomers (Satake and Sakai, 2008; Rozenfeld and Devi, 2011).

The presence or absence of an acetyl group at the N-terminal of α -MSH is involved in the regulation of this peptide's biological activities. In frogs, pigment-dispersing activities in chromatophores are enhanced by the presence of an acetyl group (Ebelre, 1988), while desacetyl (Des-Ac)- α -MSH stimulates lipid mobilization in the liver of rainbow trout (Yada et al., 2000). Moreover, diacetyl (Di-Ac)- α -MSH, which has acetyl groups at the N

and O positions of the N-terminal Ser residue, demonstrates substantial cortisol-releasing activity on the head kidney in tilapia (Lamers et al., 1992). This activity is followed by Des-Ac- α -MSH and α -MSH in order of potency. Namely, these results suggest that the number of acetyl groups on α -MSH-related peptides may influence ligand–receptor interactions, and that the biological activities of these peptides are not always proportional to the degree of acetylation. These findings are also different from pharmacological properties demonstrating that the interactions of α -MSH and Di-Ac- α -MSH via solely expressed MCRs are greater than those of Des-Ac- α -MSH (Schiöth et al., 1996; Sánchez et al., 2009a,b, 2010). We have obtained evidence solving these paradoxical results using bf flounder as well as other fish such as Jpn flounder and goldfish, as described farther on.

POMC AND MC PEPTIDES IN FLOUNDERS

While most teleosts have two POMC subtypes, probably caused by gene duplication events during fish evolution, we first reported that three *pomc* subtypes are expressed in the pituitary gland of bf flounders (Takahashi et al., 2005). Two of the POMC types (A and B) contain two MC sequences corresponding to α - and β -MSH, and 1 β -END, as is the case for other teleosts. The third POMC, namely POMC-C, is composed of α - and β -MSH, and a β -END remnant, which appears to be secondarily mutated after divergence from the strain leading to POMC-A and -B. Therefore, it is conceivable that POMC-C may have lost the bifunctional role as a precursor for both MC peptides and β -END, and became specialized for the precursor of MC peptides, the so-called promelanocortin, as we previously mentioned (Takahashi et al., 2005; Takahashi and Kawauchi, 2006a). While the amino acid sequence of α -MSH – including α -MSH-A and -B derived from bf flounder POMC-A and -B, respectively – is most conserved among the several MSH types, bf flounder α -MSH-C derived from POMC-C differs by one residue with respect to the sequences of α -MSH – at position 13 (Thr-amide in α -MSH-C vs. Val-amide α -MSH; Takahashi et al., 2005).

Pomcs are expressed in both the PD and NIL of the bf flounder pituitary gland (Takahashi et al., 2006). In these lobes, POMCs are cleaved into several biologically active peptides. The major products in the PD are ACTH, while a part of ACTH is further cleaved into Des-Ac- α -MSH, and those in the NIL are Des-Ac- α -MSH, α -MSH, Di-Ac- α -MSH, β -MSH, and C-terminally truncated N-Ac- β -END. Thus, the differences between the PD and NIL reside in the final products, including the occurrence of acetylation.

The *pomc-a* transcripts are most predominant in the pituitary, where *pomc-b* transcripts are second in predominance (Kobayashi et al., 2009). While *pomc-c* is expressed in the pituitary, this gene is also expressed in the eyed side and non-eyed side skin of the flounder. While *pomc-c* transcripts are detected at extremely low concentrations in the skin compared to that in the pituitary, their total amounts in whole skins are comparable to those of *pomc-a* in whole pituitary glands. Des-Ac- α -MSH-C derived from POMC-C is also observed from bf flounder skin extracts, indicating that the skin is another major source of α -MSH-related peptides.

Pomc-a, *pomc-b*, and *pomc-c* cDNA have been cloned in Jpn flounder (Kim et al., 2009 and accession number ACG68732); the original terms POMC-II, POMC-I, and POMC-III in the paper

correspond to POMC-A, POMC-B, and POMC-C, respectively. We observed the expression of these three genes in the pituitary and those of *pomc-c* in Jpn flounder skin. Therefore, it is indicated that the *pomc* expression patterns of these two flounder species are closely related.

Mcr SUBTYPES IN FLOUNDERS AND GOLDFISH

The presence of *mcr* has been reported in wide vertebrate classes such as Cephalaspidomorpha (lampreys), Chondrichthyes (sharks), Sarcopterygii (lobe-finned fish, including tetrapods), and Actinopterygii (ray-finned fish; Klovin et al., 2004; Schiöth et al., 2005; Haitina et al., 2007a,b). While mammals and chickens have shown to possess five *mcr* subtypes – *mc1r* to *mc5r*, zebrafish is the only fish species in which the presence of a set of five subtypes was demonstrated, while its *mc5r* is subdivided into *mc5ra* and *mc5rb* (Logan et al., 2003; Klovin et al., 2004). *Fugu* may lack *mc3r*, while four *mcr* subtypes have been identified by genomic studies. We have cloned the cDNAs for *mc1r*, *mc2r*, *mc4r*, and *mc5r* in bf flounder (Kobayashi et al., 2008, 2010, 2011). These four *mcr* cDNAs have also been identified in Jpn flounder (Kobayashi et al., 2012). Taking the taxonomically close relationship between Pleuronectiformes, including flounders, and Tetraodontiformes, including *Fugu*, into consideration, flounders may lack *mc3r*, as is the case with *Fugu*.

On the other hand, we recently demonstrated the presence of *mc1r*, *mc2r*, and *mc3r* in goldfish (Kobayashi et al., 2011). These results, as well as the previous characterization of *mc4r* and *mc5r* cDNA (Cerdá-Reverter et al., 2003a,b), provide the second line of evidence for the presence of five *mcr* subtypes in fish. The Cypriniformes, to which both goldfish and zebrafish belong, is a group of ray-finned fish that is rather basal when compared to the *Fugu* of Tetraodontiformes (Nelson, 2006). Therefore, it is possible that the five *mcr* subtypes may have appeared in an early vertebrate, possibly in a common ancestor of ray-finned fish and tetrapods. *Mc3r* may have been deleted during the course of evolution from a strain from which ray-finned fishes such as flounder and *Fugu* were descended.

α -MSH ACTIVITY: RELATIONSHIP WITH ACETYLATION AND *mcrs* EXPRESSED IN CHROMATOPHORES

NEW CONCEPTS OBTAINED FROM bf FLOUNDER

In teleost fish, MSHs stimulate not only melanophores but also other bright-colored chromatophores – erythrophores, xanthophores, and leucophores (Fujii and Oshima, 1986; Fujii, 2000). In classical concepts, acetylation increases the melanin-dispersing activity of α -MSH in teleosts (Kawauchi et al., 1984) as well as amphibians (Ebelre, 1988). Moreover, α -MSH-related peptides stimulate both melanophores and xanthophores in bf flounder; however, N-terminal acetylation differentially modulates the pigment-dispersing activities of these cells (Kobayashi et al., 2009, 2010). Surprisingly, acetylation reduces the activity of α -MSH on melanophores, while enhancing its activity on xanthophores (Table 1). Reduction of the pigment-dispersing activity of α -MSH by acetylation is the first piece of evidence for this. On the other hand, replacement of the C-terminal residue of α -MSHs may have negligible effects on pigment-dispersing activities because Des-Ac- α -MSH-C and Des-Ac- α -MSH exhibit similar effects on the two chromatophore types (Kobayashi et al., 2009). We expect the differences in the effects of α -MSHs on pigment dispersion with modification of the N-terminal residue to provide insight into the interaction of α -MSH-related peptides with MCRs.

In the early stage of our investigation into MC systems in fish skin, preliminary findings detected the expression of *mc1r*, *mc4r*, and *mc5r* in bf flounder skin parts (Kobayashi et al., 2007). Based on these results, we assumed that different pigment-dispersing activities between Des-Ac- α -MSH and α -MSH were results from the difference in MCR subtypes generated between xanthophores and melanophores. In brief, MCR(s) on xanthophores might interact with α -MSHs irrespective of the presence or absence of an acetyl group, demonstrating higher sensitivities to α -MSH than to Des-Ac- α -MSH. With the exception of MC2R, a specific receptor of ACTH, α -MSH has demonstrated the highest affinity to MC1R among the MCRs in pharmacological studies using human MCRs (Schiöth et al., 1997). Moreover, Des-Ac- α -MSH demonstrated a higher affinity to MC4R and MC5R than to MC1R

Table 1 | *Mcr* subtypes expressed in fish chromatophores and pigment-dispersing activities of α -MSH-related peptides in these cells.

Chromatophores	<i>Mcr</i> subtypes	Pigment-dispersing activities		
		Des	Mono	Di
BARFIN FLOUNDER¹				
Melanophores	1 and 5	+	–	N
Xanthophores	5	+	++	N
JAPANESE FLOUNDER²				
Melanophores	1 and 5	+	–	+
Xanthophores	5	+	+	+
GOLDFISH³				
Xanthophores	1	+	++	++

Des, Des-Ac- α -MSH; Mono, α -MSH; Di, Di-Ac- α -MSH; N, not examined.

¹Taken from Kobayashi et al. (2010).

²Taken from Kobayashi et al. (2012).

³Taken from Kobayashi et al. (2011).

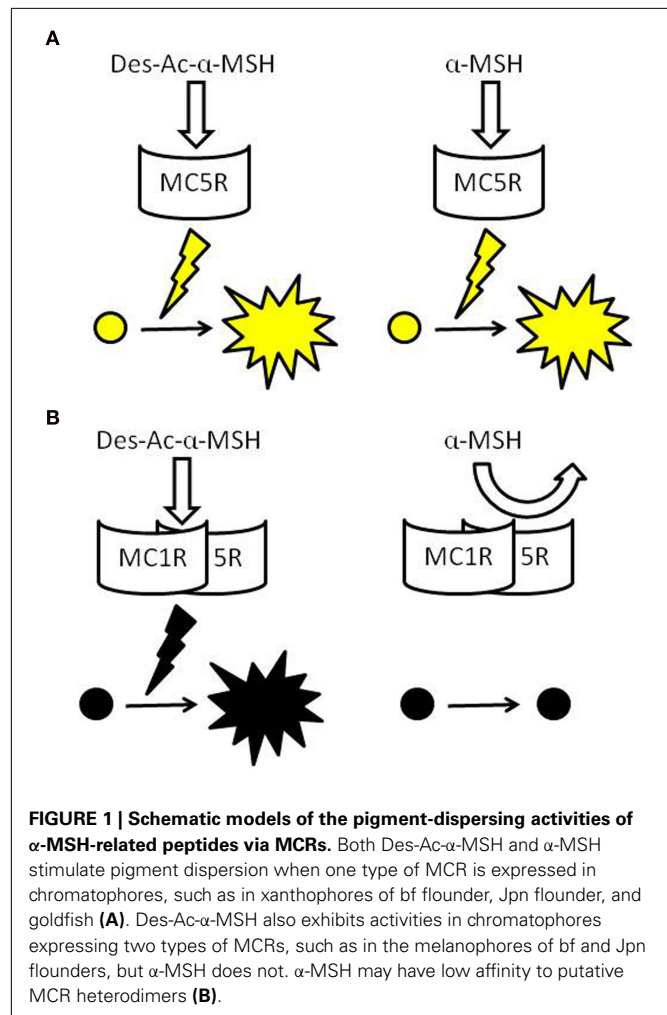
in humans (Schiöth et al., 1997). Therefore, we assumed that a receptor(s) similar to human MC4R and/or MC5R is generated in the melanophores of bf flounder skin. To verify this assumption, we examined *mcr* expression using isolated skin cells. The results indicated that we needed to change our assumption because modulation of the pigment-dispersing activity was suggested to be associated with two different *mcr* subtypes expressed concomitantly, as described below.

Two chromatophore types, melanophores and xanthophores, are observed in bf flounder skin. These chromatophores and non-chromatophoric dermal cells were isolated from skin pieces and used to examine *mcr* expression. When RT-PCR was performed for *mc1r*, *mc2r*, *mc4r*, and *mc5r* using total RNA extracted from single cells of bf flounder fin skin, a cDNA fragment of *mc5r*, but not those of other subtypes, was amplified from the total RNA prepared from xanthophores (Table 1). As described above, α -MSH exhibited higher pigment-dispersing activity than Des-Ac- α -MSH did, suggesting that acetylation at the N-terminus of α -MSH increased the activity. Pharmacological studies on sea bass MC5R have revealed that α -MSH efficacy in stimulating cellular activities is higher than that of Des-Ac- α -MSH (Sánchez et al., 2009a). Acetylation-mediated augmentation of the binding affinity of Des-Ac- α -MSH was also observed during pharmacological studies with human MC5R (Schiöth et al., 1997). Therefore, it is possible that acetylation of the ligand results in increased binding affinity of the ligand to MC5R, thus leading to increased pigment-dispersing activity.

In contrast, α -MSH did not stimulate pigment dispersion in melanophores (Kobayashi et al., 2009). When RT-PCR was performed using total RNA extracted from single cells of bf flounder fin skin, cDNA fragments of *mc1r* and *mc5r*, but not those of other subtypes, was amplified from the total RNA prepared from melanophores (Table 1). An apparent contradiction concerning pigment migration in xanthophores could be elucidated by the expression of two different *mcr* subtypes in bf flounder melanophores. This idea came from increasing biochemical and biophysical evidences indicating that many GPCRs form dimers or higher orders of oligomers (Angers et al., 2002; Breitwieser, 2004; Kroeger et al., 2004; Nakata et al., 2005; Eglén et al., 2007; Milligan, 2007). Such receptor dimerization may occur not only between identical molecules that comprise homodimers, but also between related molecules belonging to the GPCR families. Heterodimerization may either increase or decrease the affinity of ligands to the corresponding GPCRs. For example, a heterodimer consisting of somatostatin receptor (SSTR) five and dopamine D2 receptor exhibits a high affinity for both SSTR and D2 agonists (Rocheville et al., 2000). On the other hand, a heterodimer consisting of SSTR 2A and SSTR3 inactivates SSTR3 function (Pfeiffer et al., 2001). According to these findings, it is plausible that MC1R and MC5R on bf flounder melanophores may constitute heterodimers. It is also conceivable that the affinity of α -MSH with an acetyl group may be dramatically reduced by heterodimerization of the MCRs, whereas the affinity of Des-Ac- α -MSH is retained (Figure 1).

EVIDENCE OBTAINED FROM Jpn FLOUNDER

We evaluated the results obtained with bf flounder using Jpn flounder, which is another member of Pleuronectiformes, to determine



whether relationships between the effects of α -MSH-related peptides and *mcrs* expressed in chromatophores are restricted to bf flounder or are common to other species. In other words, we investigated whether reduction of the pigment-dispersing activities of α -MSH is related to the expression of two different *mcr* types.

The expression of only *mc5r* among four *mcr* subtypes was observed in Jpn flounder xanthophores. This result is identical to that obtained in bf flounder (Kobayashi et al., 2009). Differences between the two flounders were observed in the pigment dispersion in xanthophores; namely, α -MSH activities were indistinguishable from those of Des-Ac- α -MSH in Jpn flounder, while α -MSH was more potent than Des-Ac- α -MSH in bf flounder (Kobayashi et al., 2009). Thus, the pigment-dispersing activity of α -MSH is not always enhanced by the presence of an acetyl group in chromatophores expressing a single *mcr* subtype, while pharmacological studies using sea bass MC5R expressed in human embryonic kidney (HEK) 293 cells have demonstrated that the response to α -MSH is undoubtedly higher than the response to Des-Ac- α -MSH (Sánchez et al., 2009a). These results suggest that signal transduction processes in relation to pigment migration between the xanthophores of bf and Jpn flounders are different, while *Mc5r* is expressed in these cells.

Mcl1r and *mc5r* transcripts were detected in Jpn flounder melanophores. This result is the same as that obtained from bf flounder (Kobayashi et al., 2010). The effects of α -MSH-related peptides on pigment dispersion in Jpn flounder were also comparable to those observed in bf flounder; namely, Des-Ac- α -MSH exhibited the activities, while α -MSH never stimulated pigment dispersion (Kobayashi et al., 2009). Consequently, experiments using Jpn and bf flounders demonstrated that the remarkable reduction of pigment-dispersing activity is observed specifically in melanophores, in which two different *mcr* types are concomitantly expressed. Therefore, it is possible that the physicochemical property of α -MSH originating from the presence of an acetyl group causes the reduction of its activity, and this reduction appears to be associated with melanophore properties that differ from that of xanthophores with regard to ligand–receptor interaction. Results obtained from Jpn flounder support the occurrence of MCR heterodimers modifying the activity of α -MSH-related peptides (Figure 1).

EVIDENCE OBTAINED FROM GOLDFISH

The pigment-dispersing activity of α -MSH in goldfish xanthophores is somewhat greater than that of Des-Ac- α -MSH. These results are similar to those observed in bf flounder xanthophores (Kobayashi et al., 2009). Only one *mcr* subtype, i.e., *mcl1r* and *mc5r* in goldfish and flounders, respectively, is expressed in the xanthophores. Considered together, α -MSH-related peptides exhibit pigment-dispersing activities irrespective of the degree of acetylation when one type of *Mcr* is expressed (Table 1). Monoacetylation

of the N-terminus may contribute to an increase in the pigment-dispersing activity of α -MSH-related peptides in goldfish because the α -MSH activity was found to be slightly but significantly higher than that of Des-Ac- α -MSH when their effects were compared at low concentrations. Similar enhancing effects of pigment dispersion caused by monoacetylation have also been observed in grass carp, tilapia, and frogs (Kawauchi et al., 1984; Ebelre, 1988; van der Salm et al., 2005). Only one *mcr* subtype appears to be expressed in the melanophores of these species. Additionally, the pigment-dispersing activity of α -MSH was comparable to that of Di-Ac- α -MSH. Pharmacological studies using sea bass MC1R expressed in HEK 293 cells have exhibited similar responses to both α - and Di-Ac- α -MSH (Sánchez et al., 2010). Similar pharmacological properties have been observed for sea bass MC5R (Sánchez et al., 2009a). Therefore, it is suggested that both MC1R and MC5R recognize α -MSH and Di-Ac- α -MSH equally.

CONCLUSION

Based on comparative studies using chromatophores from flounders and goldfish, it is hypothesized that the pigment-dispersing activity of α -MSH is reduced in chromatophores expressing two different *mcr* subtypes due to the formation of MCR heterodimers. On the other hand, when one *mcr* type is expressed, as in the case of flounder and goldfish xanthophores, α -MSH-related peptides such as Des-Ac- α -MSH, α -MSH, and Di-Ac- α -MSH exhibit pigment-dispersing activities in a dose-dependent manner irrespective of the degree of acetylation. Further experiments using a variety of fishes are necessary to confirm the hypothesis.

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