Bull. Inst. Zool., Academia Sinica **25**(2): 105-111 (1986)

ANALYSIS OF MUSCULAR PROTEINS OF TILAPIA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY[1]

JEN-LEIH WU, WEI-JEN CHAO and YA-LI HSU

Institute of Zoology, Academia Sinica,
Taipei, Taiwan 11529,
Republic of China

(Accepted March 28, 1986)

Jen-Leih Wu, Wei-Jen Chao and Ya-Li Hsu (1986) Analysis of muscular proteins of tilapia by high-performance liquid chromatography. *Bull. Inst. Zool., Academia Sinica* **25**(2): 105-111. The separation of protein mixture was performed by high-performance liquid chromatography (HPLC) with a combination of protein PAK 125 and protein PAK 300 SW columns. The effects of pH and ionic strength of different buffer systems on the separation of eight standard proteins (MW: 500-450,000) were investigated. The best buffer condition is 0.25 M phosphate buffer, pH 6.0 with a flow rate of 1 ml/min. The elution pattern of muscular protein of tilapia was also analyzed in these HPLC columns. The calculation of molecular weight of each muscular protein was based on standards elution curve. The relative amount of each muscular protein was also determined.

Key words: ***HPLC, molecular weight, muscular protein, Tilapia.***

Tilapia become one of the most important fresh water aquaculture in Taiwan, its production is next to milkfish's (Schoombee 1979, Taiwan Fisheries Bureau, 1982). The annual production of tilapia in Taiwan was increased rapidly. During the ten consecutive years, its annual productions were 13,000 tons in 1974, 22,000 tons in 1977 (Schoombee, 1979), 48,500 tons in 1981 (Taiwan Fisheries Bureau, 1982), 28,877 tons in 1982, and 30,382 tons in 1983. This steady increase of tilapia culture was due to the applications of all-male hybrids breeding between male *Oreochromis aureus* and female *O. niloticus* (Hepher and Pruginin, 1982; Pruginin *et al.*, 1975). Red tilapia *O. mossambicus* were early culture species and also spread widely. Therefore, the identification and the maintenance of pure stock are the basic prerequisite for tilapia culture. The morphological examination is the traditional method to identify the tilapia species. However, people were always confused by the hybrids of tilapia. Since the farmers did not pay much attention on controlling the production and the maintanence of a pure stock. Thus, the identification of the tilapia species becomes more difficult. A more accurate method for species identification need to be established.

The species identification by isozyme and protein electrophoresis has been widely appled in fish (Morgan and Ulanowicz, 1976; Yeardley and Hubbs, 1976). The specific characters of tilapia species from different organs have been fully established by esterase isozymes (Herzberg, 1978; Wu and Wu, 1983). High-

1. Paper No. 273 of the Journal Series of the Institute of Zoology, Academia Sinica.

performance liquid chromatography (HPLC) with gel permeation columns has been used in the analysis of many biological substances, such as polypeptides, proteins, lipoproteins and enzymes (Hashimoto, *et al.* 1978; Imamura, *et al.* 1979; Krstuloric, *et al.* 1979; Mabuchi and Nakahashi, 1981; Okazaki, *et al.* 1980; Ratge and Wisser, 1982). In this paper, we have investigated the effects of pH and ionic strength of different buffer systems on the separation of 8 standard proteins with an MW ranged from 500 to 450,000 in HPLC. The molecular weight of each muscular protein of tilapia was calculated from the elution pattern in HPLC with 0.25 M phosphate buffer, pH 6.0. The relative amount of each muscular protein in total tilapia muscle was also determined.

MATERIALS AND METHODS

Muscular Proteins preparation

Tilapia hybrids between male *O. aureus* and female *O. niloticus* were used. the dorsal muscle of tilapia was excised from fresh fish and homogenized with two volumes of 0.1 M Tris-HCl buffer, pH 8.0. Then the homogenate was centrifuged at 12,100 xg for 20 min, at 4°C. The supernatant was filtered through 0.45 μm filter (Millipore, Bedford, MA) and stored at 4°C for HPLC analysis.

Standard proteins and nucletide

Eight different known molecular weight proteins were separated into 2 groups. Group A protein mixture contained ferritin, 450,000; aldolase, 158,000; ovalbumin, 45,000; and cytochrome c, 12,500. Group B mixture: catalase, 240,000; albumin, 68,000; chymotrypsin, 25,000; and adenosine triphosphate. 507.

Elution Buffers

Six different concentrations and different pH of phosphate buffers were used: (1) 0.25 M phosphate buffer, pH 4.43; (2) 0.25 M phosphate buffer, pH 6.0; (3) 0.25 M phosphate buffer, pH 6.89; (4) 0.067 M phosphate buffer, pH 6.74, containing 0.3 M NaCl; (6) 0.25 phosphate buffer, pH 6.94, containing 0.1 M CH_3COONa. Two sodium acetate buffers were used: 0.15 M CH_3COONa, pH 7.89. 0.1 M ammonium acetate beffer, pH 6.94, and 0.1 M sodium sulfate buffer, pH 5.9, were used too. All of these buffers were filtered through a 0.45 μm filter (Millipore, Bedford, MA) before use.

High-performance liquid chromatography

The apparatus is consisted of a Waters Assoc. Model 680 automatic gradient controller, a Model 510 pump, a Model 441 UV detector, and a Model 730 data module. The gel filtration columns used were a sequential connection of protein PAK 125 (7.8 mm×30 cm, MW 1,000-80,000) and protein PAK 300 SW (7.5 mm×30 cm, MW 2,000-400,000).

Normally 10 μl of membrane filtered standard protein mixture (20 μg protein) or 5 μl of tilapia muscular protein solution (40 μg protein) was injected into the HPLC. The buffer system as indicated in the text was pumped throught the gel filtration columns at a flow rate of 1 ml/min. The fractions containing proteins were eluted out about 27 ml of indicated buffer, and the UV absorbance at 280 nm was used to monitor the protein peaks.

RESULTS AND DISCUSSION

Effects of different buffer systems on HPLC separation of eight standards

The effects of different buffer systems with different pH and ionic strength on the HPLC separation of 8 protein standards by using the combination columns of protein PAK 125 and protein PAK 300 SW were conducted as shown in Fig. 1. Group A protein standards could be separated into 4 peaks (A_{280}) by HPLC eluted with 0.25 M phosphate buffer, pH 6.0; pH 6.89; 0.067 M phosphate buffer, pH 6.74, containing 0.1 M KCl; and 0.05 M phosphate buffer, pH 6.45, containing 0.3 M NaCl (Fig. 1A1, 1A2, 1A3, 1A4). The separation of group A proteins by using

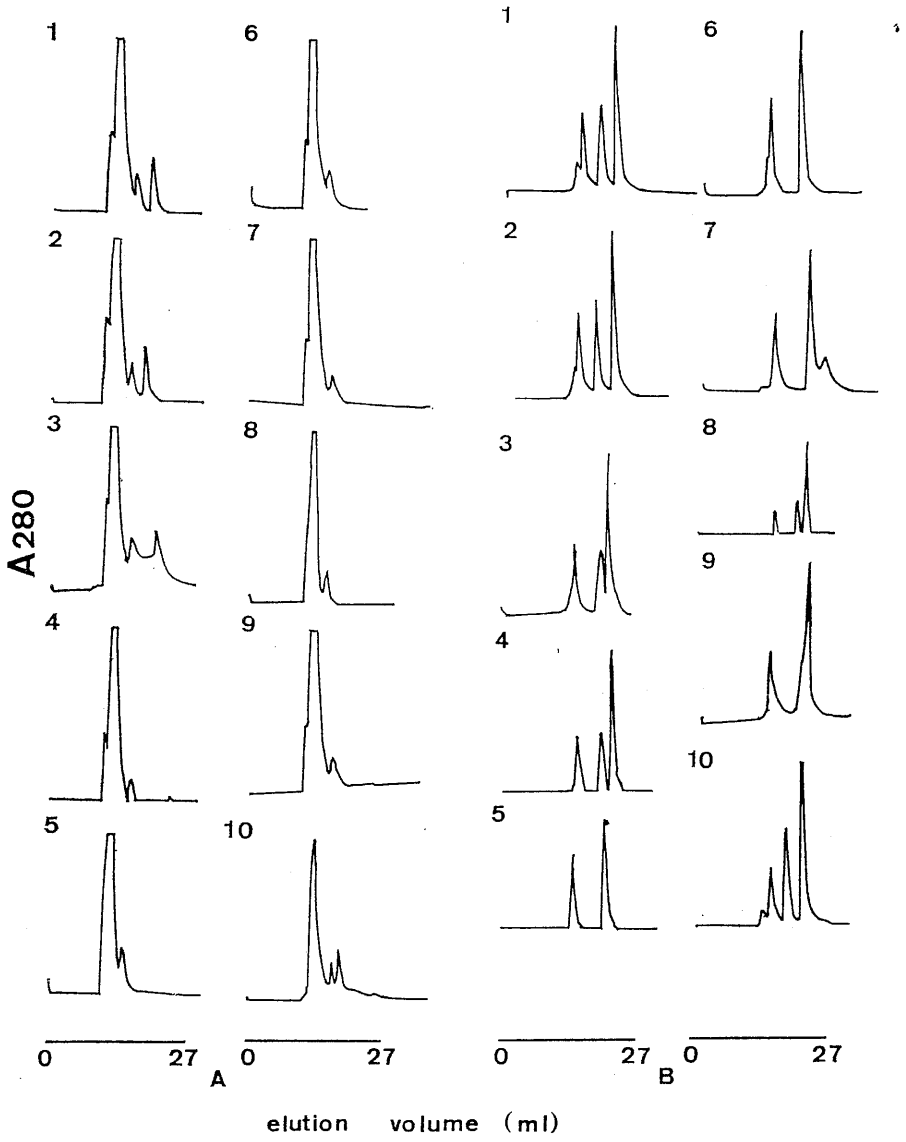

Fig. 1. HPLC elution patterns of the standard proteins (A_{280}) nucleotide with different buffers. Column, protein PAK 125+protein PAK 300 SW. Flow-rate, 1.0 ml/min.

Group A mixture: ferritin, aldolase, ovalbumin, cytochrome C.

Group B mixture: catalase, albumin, chymotrypsin, adenosine triphosphate.

Buffers:

1. 0.25 M phosphate buffer, pH 6.0.
2. 0.25 M phosphate bufferm pH 6.89.
3. 0.067 M phosphate buffer, pH 6.74+0.1 M KCl.
4. 0.05 M phosphate buffer, pH 6.45+0.3 M NaCl.
5. 0.15 M CH_3COONa, pH 6.92.
6. 0.1 M CH_3COONa, pH 7.89.
7. 0.1 M CH_3COON_4, pH 6.94.
8. 0.1 M Na_2SO_4, pH 5.9.
9. 0.25 M phosphate buffer, pH 6.94+0.1 M CH_3COONa.
10. 0.25 M phosphate buffer, pH 4.43.

acetate buffers (Fig. 1A5, 1A6, 1A7 and 1A9) and 0.25 M phosphate buffer, pH 4.43 (Fig. 1A10) was poor, since only three peaks were obtained from this HPLC columns. And merely two protein peaks (A_{280}) were observed by 0.1 M Na_2SO_4 buffer, pH 5.9.

The same ten different buffer systems were also used to separate group B standards. The results were shown in Fig. 1B. Four peaks could be eluted by 0.25 M phosphate buffer, pH 6.0; pH 6.89; and pH 4.43 (Fig. 1B1, 1B2 and 1B10). When KCl or NaCl was present in phosphate buffer (Fig. 1B3 and 1B4), the separation would become poor. This is different from the separation effect of group A proteins. The separation by using acetate buffers (Fig. 1B5, 1B6, 1B7 and 1B9) was also not good, particularly 0.15 M sodium acetate, pH 6.92 and 0.25 M phosphate buffer containing 0.1 M sodium acetate, pH 6.94. Only two protein peaks (A_{280}) could be obtained (Fig. 1B5 and 1B9). From the data shown in Fig. 1, phosphate buffer is a better eluent for separation of these proteins, and sodium acetate buffer is the worst one.

If we want to get all eight standard peaks eluted from HPLC columns, the better eluents should be 0.25 M phosphate buffer, pH 6.0 and pH 6.89.

HPLC elution patterns (A_{280}) of the muscular proteins of tilapia

By using the same columns, the HPLC elution patterns of the muscular proteins of tilapia eluted by 0.25 M phosphate buffer, pH 6.0 and pH 6.89 were shown in Fig. 2. Seven peaks were eluted out by using phosphate buffer, pH 6.0 (Fig. 2A). But only five peaks could be eluted by phosphate buffer, pH 6.89 (Fig. 2B). Therefore, pH 6.0 phosphate buffer give better separation efficiency on the muscular proteins of tilapia than pH 6.89 phosphate buffer does (Fig. 2). This result is similar to these phosphate buffers separating group B standards.

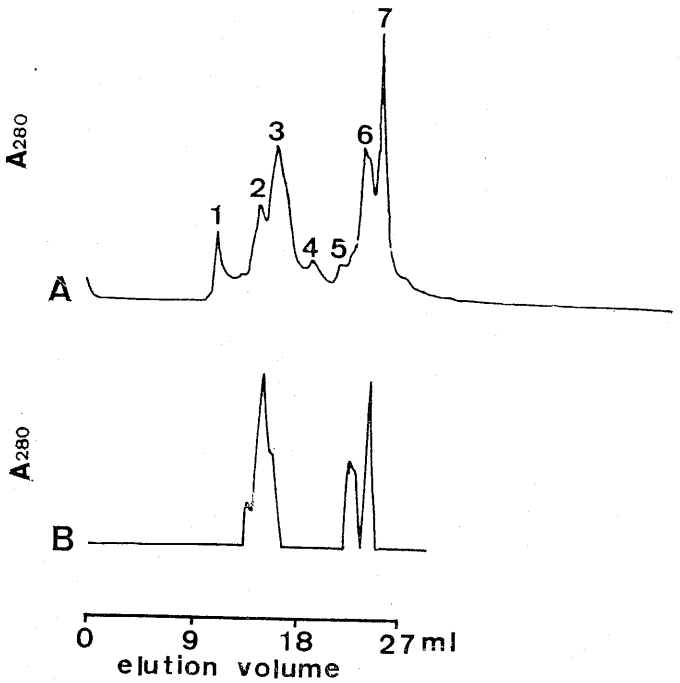

Fig. 2. HPLC elution patterns of the muscular proteins of tilapia. Column, protein PAK 125+protein PAK 300 SW; elution buffer, pH 6.0; (2) 0.25 M phosphate buffer, pH 6.89; flow rate, a ml/mun.

The relationship between molecular weight and elution volume

From the comparison of separation effects of different elution buffers on the protein standards (Fig. 1) and muscular proteins of tilapia (Fig. 2), 0.25 M phosphate buffer, pH 6.0 was the best eluent. A regression line of the eight protein standards with this eluent is plotted semilogarithmically in Fig. 3. With the combination columns of protein PAK 125 and 300 SW, the best separation efficiency is in the molecular weight from 1,000 to

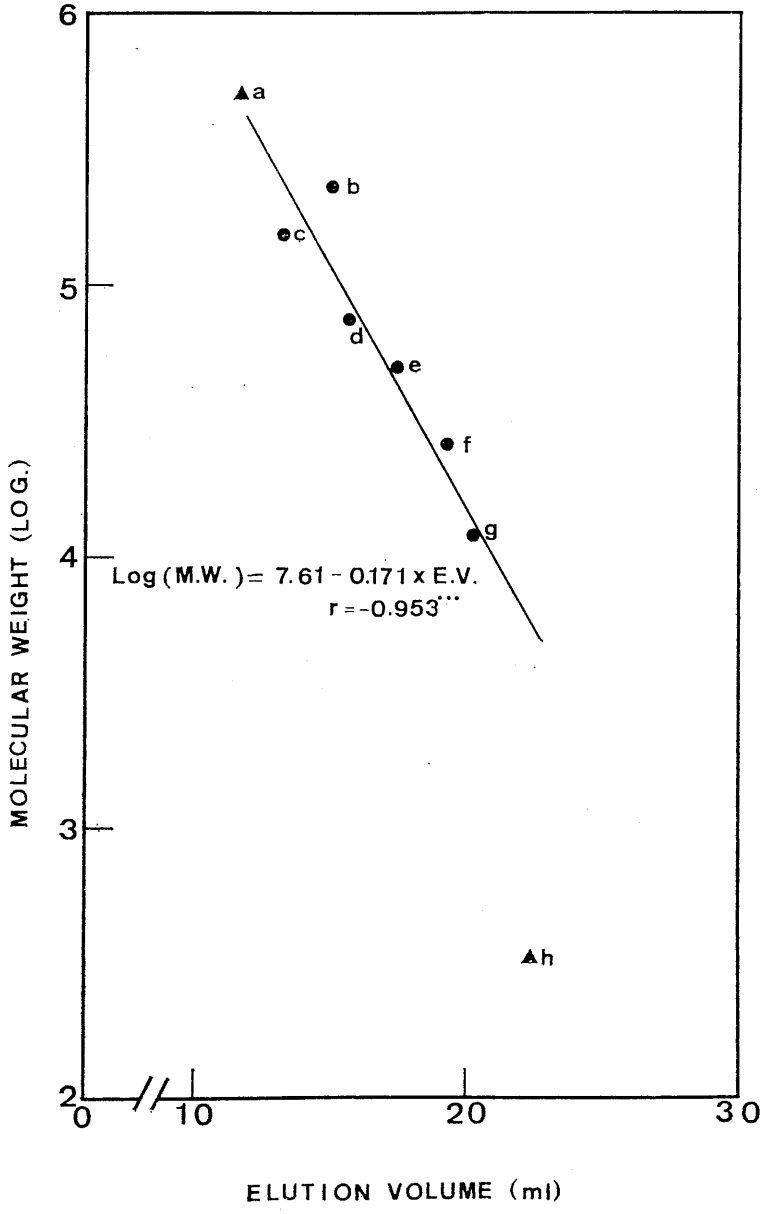

Fig. 3. Relationship between molecular weight and elution volume in HPLC. Elution conditions were the same as Fig. 1. Reference compounds: a, Ferritin (450,000); b, Catalase (240,000); c, Aldolase (158,000); d, Albumin (68,000); e, Ovalbumin (45,000); f, Chymotrypsin (25,000); g, Cytochrome c (12,500), and h, ATP (507).

400,000. Therefore, ATP (507) and ferritin 450,000) are out of the range (1,000 to 4000,000). A linear response between the molecular weight of the other six protein standard and elution volume was shown in Fig. 3. The regression formula of molecular weight (log) is equal to 7.76-0.171× (elution volume). $r=-0.953$. The linear relationship between molecular weight and elution volume for HPLC G 3000 SW columns (Ratge and Weisser, 1982), TSK 4000 SK and a combination of TSK 4000 PW and TSK 3000 PW columns (Williams, *et al.*, 1984), TSK G-SW and Synchropak GPC columns (Shimohigashi *et al.*, 1983) has been investigated.

The molecular weight and the relative proportion of each muscular protein of tilapia

Seven protein peaks of the tilapia muscle could be eluted out by the phosphate buffer, 0.25 M, pH 6.0 from HPLC columns (Fig. 2A) From the linear regression in Fig. 3, Log (MW) = 7.61 − 0.171 × elution volume, the molecular weight of each protein of tilapia muscle can be estimated; peak 1, 400,000; peak 2, 143,000; peak 3, 88,300; peak 4, 24,100; peak 5, 8,000; peak 6, 4,200; peak 7, 1,000 (Fig. 2A and Table 1). Since the ATP, a nucleotide, can also absorb the light at 280 nm, peak 7 is a small molecule, and also could be a nucleotide or other small biomolecules. The usefulness of gel HPLC with the combination of columns protein PAK 125 and protein PAK 300 SW in size fractionation and molecular weight determination has been demonstrated. The advantages of these procedures over the conventional soft-gel permeation columns are dramatic reduction in both analysis time and sample size, and increased reproducibility. The relative amount of each muscular protein peak in Fig. 2A can be estimated (Table 1). Peak 7 has the highest quantity, peak 3 is the next, and peak 6 is the third. In conclusion, from the HPLC protein elution pattern of tilapia muscle, we can estimate the moleular weight and relative quantity of each protein peak of tilapia muscle. By using these two criteria, different tilapia species might be distinquished. However, further study should be done before using the HPLC protein elution pattern as the genetic marker of tilapia.

TABLE 1
The molecular weight and relative absorbancy of muscular proteins of tilapia

Peak number[a]	Molecular weight[b]	Relative absorbancy[c] (%)
1	400,000	6.52
2	143,200	12.00
3	88,300	26.16
4	24,100	6.10
5	8,800	3.25
6	4,200	17.74
7	1,000	28.24

a. Peak number is labeled in Fig. 2A, from left to right.
b. Molecular weight were determined as described in Results and Discussion.
c. Percentage of each protein was calculated from the area under each peak of muscular protein of Tilapia in HPLC analysis (Fig. 2A).

Acknowledgements: The financial support from council of Agriculture, Republic of China, is gratefully acknowledged.

REFERENCES

HASHIMOTO, T., H. SASAKI, M. AIURA and Y. KATO (1978) High speed aqueous gel-permeation chromatography of proteins. *J. Chromatogr.* **160**: 301.

HEPHER, B. and Y. PRUGININ (1982) Tilapia culture in ponds under controlled conditions. In the biology and culture of tilapias (R. S. V. Pullin and R. H. Lowe-McConnell eds.). ICLARM conference proceedings, Manila, Philippines. 186pp.

HERZBERG, A. (1978) Electrophoretic esterase pattern of the surface mucus for the identification of *Tilapia* species. *Aquaculture* **13**: 81-83.

IMAMURA, T., K. KONISHI, M. YOKOYAMA and K. KONISHI (1979) High-speed gel filtration of polypeptides in sodium dodecyl sulfate. *J. Biochem.* **86**: 639-642.

KRSTULORIC, A. M., R. A. HARTWICK and P. R. BROWN (1979) High-performance liquid chromatographic assay for acid and alkaline phosphatase in serum. *J. Chromatogr.* **163**: 19.

MABUCHI, H. and H. NAKAHASHI (1981) Analysis of middle molecular peptides in normal and uremic body fluids by high-performance gel chromatography. *J. Chromatogr.* **224**: 322.

MORGAN, R. P. and N. I. ULANOWICZ (1976) The frequency of muscle protein polymorphism in *Menidia menidia* (Atherinidae) along the Atlantic coast. *Copeia* **1976**: 356-360.

OKAZAKI, M., Y. OHNO and I. HARA (1980) High-performance aqueous gel permeation chromatography of human serum lipoproteins. *J. Chromatogr.* **221**: 257.

PRUGININ, Y., S. ROTHBARD, G. WOHLFARTH, A. HALEVY, R. MOW and G. HULATA (1975) All male broods of Tilapia nilotica × T. aurea hybrids. *Aquaculture* **6**: 11-21.

RATGE, D. and H. WISSER (1982) Urinary protein profiling by high-performance gel permation chromatography. **230**: 47-56.

SCHOOMBEE, H. J. (1979) Report on visit to Taiwan Fisheries Research Institute, May-June.

SHIMOHIGASHI, Y., R. LEE and H. C. CHEN (1983) Gel high-performance liquid chromatographic studies on the elution behavior of chemically deglycosylated human chorionic gonadotropin and its subunits. *J. Chromatogr.* **266**: 555-565.

Taiwan Fisheries Bureau (1982) Fisheries Yearbook Taiwan Area. 27pp.

WILLIAMS, M. C., J. L. KELLEY and R. S. KUSHWANA (1984) Detection of an abnormal lipoprotein in a large colony of pedigreed baboons using highperformance gel exclusion chromatography. *J. Chromatogr.* **308**: 101-109.

WU, J. L. and S. Y. WU (1983) Electrophoretic differences of esterase isozymes from the surface mucus of *Oreochromis* fishes. *Bull. Inst. Zool., Academia Sinica* **22**: 133-140.

WU J. L., J. C. HSU and S. K. LOU (1983) Esterase isozymes in *Oreochromis niloticus O. aureus, O. mossambicus* and red tilapia. International symposium on Tilapia in Aquaculture. p. 281-290.

YEARDLEY, D. and C. HUBBS (1976) An electrophoretic study of two species of mosquitofish with notes on genetic subdivision. *Copeia* **1976**: 117-120.

高壓液相色層分析儀作吳郭魚肌肉蛋白質分析

吳金洌　趙偉真　徐亞莉

蛋白質混合液的分離是利用蛋白質 PAK 125 和 300 SW 管柱串聯而成的，利用不同 pH 和離子濃度的緩衝液對 8 種標準蛋白質和核苷酸（分子量由 500 到 450,000）的分離效果作分離系統之建立，其中以 pH 6.0，0.25 M 磷酸溶液；流速爲 1 ml/min 所得分離效果最好。吳郭魚肌肉蛋白質運用此高壓液相色層 (HPLC) 管柱系統作分析，每一肌蛋白的分子量是由標準曲線計算。共有七個不同分子量的部分可以分離出，分子量分別是 400,000，143,200，88,300，24,100，8,800，4,200 及 1,000 道爾頓。每部分蛋白質之相對含量予以計算其百分比，以供不同品系之吳郭魚肌肉蛋白質分析比較。