

Research Article

Isolation, Purification and Identification of Parvalbumin Allergen in *Cyprinus carpio*

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Abstract: Parvalbumin (PV) is a major fish allergen that is characterized by high water solubility, low molecular weight (i.e., ~12,000), acidic isoelectric point, heat stability and high phenylalanine content (compared with tyrosine and tryptophan). We used a fast, simple and efficient method to purify PV from *Cyprinus carpio* (carp). Heating, two runs of trichloroacetic acid precipitation and gel filtration chromatography were integrated in this method. Results of sodium dodecyl sulfate polyacrylamide gel electrophoresis and western blot showed that the purified protein was PV and the purity of carp PV obtained using this method was more than 90%. Moreover, the PV was characterized by MALDI-TOF-TOF MS and an unusually high 260 to 280 nm run absorbance ratio in the final purification stages. The rapid purification method used in this study resulted in better purification and identification of PV compared with other methods. Further studies on PV detection in freshwater fish can be performed based on the results of this experiment.

Keywords: Allergen, *Cyprinus carpio*, identification, parvalbumin, purification

INTRODUCTION

The importance of fish as a food source has increased because of its high consumption and dietary value. However, fish is also one of the most frequent causes of food allergy, especially in coastal areas and fish-processing communities (Elsayed, 2002; Sampson, 2003). Fish allergy in children and young adults is an immunoglobulin E-mediated disease with clinical manifestations ranging from urticaria and angioedema to anaphylaxis (Aas, 1966; Hansen *et al.*, 1997). Aas (1987) reported that 1/1000 of the general population in Norway is hypersensitive to fish. Sampson (1999) found that the prevalence of fish and shellfish allergy in the United States is 0.5% of the total number of patients that show hypersensitivity to food items. This value corresponds to approximately 2% of the adult population. PV is a calcium-binding sarcoplasmic protein with a molecular mass of 12 kDa. Studies on various fish species including *Cyprinus carpio* (carp) (Bugajska-Schretter *et al.*, 1999; Swoboda *et al.*, 2002) salmon (Lindstrøm *et al.*, 1996), cod (Elsayed and Aas

1971a, b; Elsayed and Bennich, 1975), horse mackerel (Shiomi *et al.*, 1998), mackerel (Hamada *et al.*, 2003) and tuna (Shiomi *et al.*, 1999) have shown that Parvalbumin (PV) is a major fish allergen. The allergenicity of fish PVs is reduced to some extent when their bound calcium is released (Swoboda *et al.*, 2002). However, PV is very stable in the presence of heat and extreme pH, under denaturing agent treatment and proteolytic enzyme treatment (Aas, 1969; Elsayed and Bennich, 1975; Bernhisel-Broadbent *et al.*, 1992; Pascual *et al.*, 1992; Elsayed and Apold, 1983).

We aimed to achieve the following objectives:

- Isolate and purify PV to high purity PV from carp through a fast, simple and efficient method.
- Identify PV with western blot.
- Characterize PV through Matrix-Assisted Laser Desorption Ionization Time-of-Flight-Time-of-Flight Mass Spectrometry (MALDI-TOF-TOF MS) and characteristic ultraviolet spectrum.
- Provide a more complete understanding of the PV allergen.

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MATERIALS AND METHODS

Fish: Live carp specimens were purchased from the Hangzhou market in Xiasha. The white muscle samples collected from these fish were stored at -20°C until use.

Heated extract preparation: The carp muscular tissue was homogenized with 5 volumes of extraction buffer (w/v, 10 mM CaCl₂, 10 mM Tris-HCl, pH 7.5). Crude protein extracts were obtained by centrifugation at 12,000 rpm for 20 min. Precipitation removal was followed by homogenate heating in boiling water bath for 15 min. The solution was then cooled to room temperature and centrifuged at 12,000 rpm for 20 min. The supernatant obtained was used as the heated extract (Guo *et al.*, 2012).

Parvalbumin purification: The Trichloroacetic Acid (TCA) method was used to purify PV from the carp muscular tissue. Phenylmethylsulfonyl fluoride (100 Mm) was added to the supernatant and the mixture was centrifuged twice at 10,000 rpm for 20 min. Consequently, 100% TCA was slowly added to the supernatant until the final TCA concentration reached 3%. The mixture was stirred for 10 min at 4°C. The pH value was adjusted to 5.2 using 6 N NaOH and the solution was again gently stirred for 1 h at 4°C. The supernatant was obtained by centrifugation at 8,000 rpm for 20 min and 3% TCA was added. The obtained precipitate was dissolved in distilled water (Zheng *et al.*, 2012). The samples underwent gel filtration on a Sephacryl-200 column (1.6-70 cm; GE Healthcare, Buckinghamshire, UK) eluted with 0.15 M NaCl-0.01 M phosphate buffer (pH 7.0) at approximately 1 mL/min flow rate. Fractions detected by a UV spectrophotometer at 280 nm were collected. Furthermore, the eluate that corresponded to each peak was manually collected and analyzed using SDS-PAGE.

SDS-PAGE: Discontinuous system vertical slab SDS-PAGE gel electrophoresis was used for this experiment. The concentrated and separated gel concentrations were 5 and 15%, respectively. The constant current was 15 mA for approximately 10 min and was changed to 25 mA thereafter. The protein standards of six peptide kinds, which ranged between 14.4 and 97.4 kDa in size, were used as references along with the samples. The gel was stained with Coomassie Brilliant Blue R-250 and bleached with acid after a 2 h run.

Protein concentration determination: In the present study, we used the Bradford method (Bradford, 1976) to determine the protein concentration and the standard curve was plotted. The absorbance of the standard protein solution with different concentrations was measured at a 595 nm wavelength by using a UV spectrophotometer. The absorbance (OD₅₉₅) was the vertical axis standard, whereas the protein content (µg/mL) was the abscissa.

Western blot: Western blot was carried out as described by Towbin *et al.* (1979). Briefly, proteins on polyacrylamide gels were electrophoretically transferred onto Polyvinylidene Fluoride (PVDF) membranes. Then the PVDF membranes were blocked with 5% skim milk. After washing with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 8.0), the membranes were incubated with anti-mouse PV monoclonal antibodies and Horseradish Peroxidase (HRP)-labeled rabbit anti-mouse IgG. Finally, the immunoassay was carried out using DAB.

MALDI-TOF-TOF MS: The target protein band was manually excised from the stained gels. They were then washed and digested with sequencing-grade trypsin (Promega, Madison, WI, USA). MALDI-TOF spectra were calibrated using trypsin autodigestive peptide signals and matrixion signals. Peptide masses were searched against the non-redundant sequence database (NCBI nr v20130918) to identify the protein using the MASCOT program (<http://www.matrixscience.com>). Search parameters were set, with the tolerance to 100 ppm peptide mass variance for carbamidomethyl (C) as fixed Modification and oxidation (M) as variable modification. For the MASCOT search results, protein scores were considered significant (p<0.05) in this experiment when greater than 45.

Absorption spectra: A Bio-Rad UV detector was used to obtain the ultraviolet PV spectrum during 200-350 nm.

RESULTS AND DISCUSSION

Pretreatment effect: PVs have high thermal stability and most of the proteins underwent denaturation precipitation after heating. Thus, the extraction of fish sarcoplasmic protein in this study was subjected to a short heat treatment to acquire high PV purity. Heat treatment removed other lipid fat and substances in the sarcoplasmic proteins and prevented interferences in subsequent detections.

Lane 2 in Fig. 1 shows that the crude extracts contained more than 15 kinds of proteins with complex components. The crude extract concentration in the Coomassie Brilliant Blue assay was approximately 5.231 mg/mL, whereas that of the heated extracts is 1.362 mg/mL. Lane 3 in Fig. 1 illustrates that the contaminating proteins were reduced considerably after boiling and centrifugation. The separation and purification steps were reduced and the results could be improved. The protein in lane 5 was greatly enriched after the second TCA precipitation.

Most previous studies indicated that PV could be purified by using the ammonium sulfate precipitation method, which is too complex. Accordingly, the samples must be dialyzed overnight after the ammonium sulfate processing steps. This procedure was time consuming and the obtained purification

Table 1: Purification procedures of carp parvalbumin allergen

Step	Total protein (mg) ^a	Protein (mg/mL) ^b	Yield (%)
Crude extract	135.6±0.31	5.231±0.012	100
After heat and filtration	27.12±0.46	1.362±0.023	20
After first TCA step	20.31±0.32	1.024±0.016	15
After second TCA step	7.05±0.30	1.123±0.048	5.2
Sephacryl S-200 effluent	1.28±0.09	1.221±0.086	0.9

^a: Data are expressed as weight values±standard variation of three replications; ^b: Data are presented as concentration values±standard variation of three replications

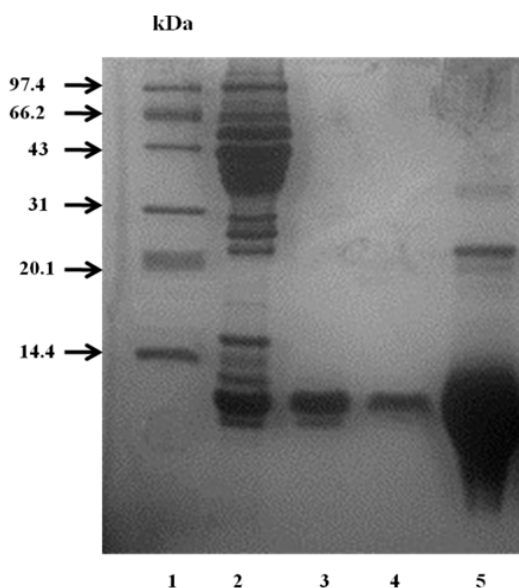


Fig.1: SDS-PAGE of the purified preparations from carp
Lane 1: Standard protein; Lane 2: Carp crude extract;
Lane 3: Carp heated extract; Lane 4: First TCA
precipitation extract; Lane 5: The second TCA
precipitation extract

effects were minimal. In the present study, we purified PV by running the TCA precipitation method twice. This approach was simple and fast and the purification effect was satisfactory. A high PV purity was obtained in SDS-PAGE after applying the TCA purified method twice.

Changes in protein: The purification summary is given in Table 1. The changes of various parameters during the purification steps are presented in the same table.

Gel filtration chromatography of SDS-PAGE: The material to be separated in gel filtration chromatography was diffused into the gel within the pores at different speeds according to molecular size, shape and type and by using a speed that was different from that used in column chromatography.

The fractions were collected and detected using a UV spectrophotometer at 280 nm after the Sephacryl S-200 gel filtration chromatography. Two peaks detected by absorbance at 280 nm were observed at fractions 36 and 53 (Fig. 2). The second peak was the main peak

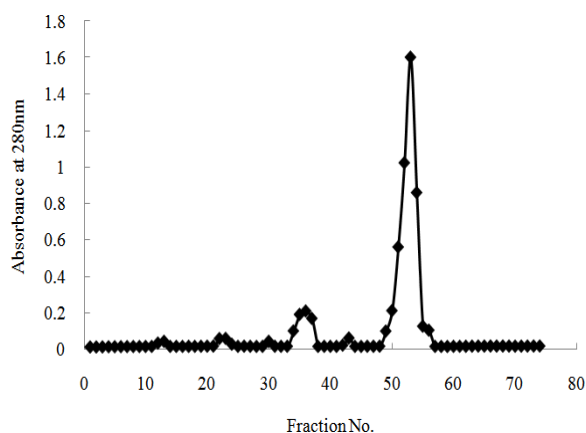


Fig. 2: Sephacryl-200 gel filtration chromatography of parvalbumin derived from carp muscle

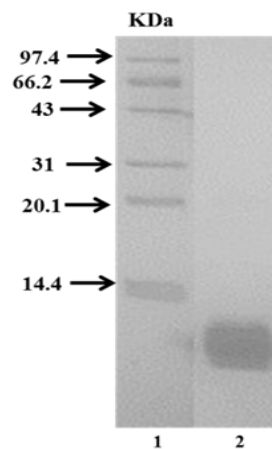


Fig. 3: SDS-PAGE of purified PV
Lane 1: Standard proteins; Lane 2: The peak II in
Fig. 2

from the absorbance. The SDS-PAGE analysis revealed that high protein purity was obtained from peak II. By the Quantity One analysis, after gel filtration chromatography, the protein had high purity (i.e., purity >90%) (data not shown).

Protein purity testing methods: SDS-PAGE primarily distinguishes proteins according to molecular weight. When Coomassie Brilliant Blue staining was used after SDS-PAGE, a single band of the protein electrophoresis detection limit was obtained at 0.1 µg. This result indicated that the protein was not detected when its content was less than 0.1 µg. The Quantity One analysis

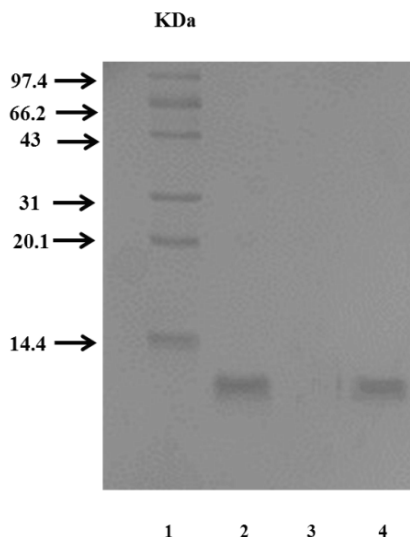


Fig. 4: Analysis of the purified parvalbumin by western blot
Line 1: Standard proteins; Lane 2: Purified parvalbumin from carp; Lane 3: Control; Lane 4: Crude extract from carp

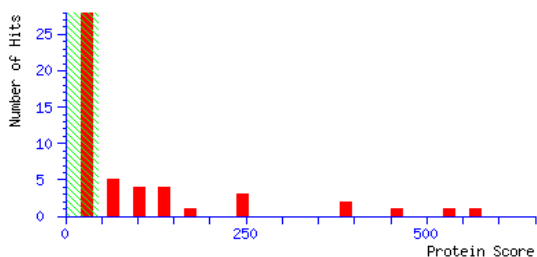
showed only one band in lane 2 of Fig. 3. Therefore, determination of high purity of the separation protein was possible (Fig. 4).

Western blot: Analysis by western blot showed that anti-mouse PV monoclonal antibodies displayed positive IgE reactivity to both the crude extract from carp and the purified protein in Fig. 5, so the purified protein is PV. No antibody binding was detected with the control groups which remove the incubation with anti-mouse PV monoclonal antibodies.

MALDI-TOF-TOF MS analysis: The successful identification was characterized by a protein score of 567 (Fig. 6). The Gi number of the protein is 17977827. The taxonomy is *Cyprinus carpio*. The protein molecular weight was 11619, whereas the isoelectric point was 4.77. The molecular mass and the isoelectric point is in agreement with its migration in SDS-PAGE and corresponds to the molecular weight deduced from the amino acid sequence of carp parvalbumin (11489 kDa) determined by Coffee and Bradshaw (1973).

Mascot Score Histogram

Ions score is $-10 \cdot \log(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 45 indicate identity or extensive homology ($p < 0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Query	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Score	Expect	Rank	Unique	Peptide
✓ 2	909.5146	908.5074	908.5120	-5.07	0	48	0.022	1		K.LFLQNFK.A
✓ 19	1020.5351	1019.5278	1019.5287	-0.91	0	84	9.5e-06	1		K.AFAIIDQDK.S
✓ 31	1148.6278	1147.6205	1147.6237	-2.77	1	44	0.069	1		K.KAFAIIDQDK.S
✓ 34	1166.5571	1165.5498	1165.5503	-0.38	0	80	2e-05	1		K.SGFIEDELK.L
✓ 38	1191.6570	1190.6497	1190.6547	-4.17	0	77	3.5e-05	1		K.IGVDEFTALVK.A
✓ 39	1198.6678	1197.6605	1197.6758	-12.73	1	33	0.65	1	U	K.TFFAKVGLTSK.S
✓ 55	1264.6809	1263.6736	1263.7088	-27.81	1	15	68	1		K.LFLQNFKAGAR.A
✓ 127	1993.9604	1992.9531	1992.9640	-5.47	1	82	1.3e-05	1		K.AGDSGDGKIGVDEFTALVK.A
✓ 140	2057.0054	2055.9981	2056.0517	-26.05	1	45	0.063	1		K.SGFIEDELKFLQNFK.A
✓ 151	2168.0552	2167.0479	2167.0685	-9.47	1	59	0.0024	1		K.AFAIIDQKSGFIEDELK.L

Fig. 5: Purified carp PV identification

Protein sequence coverage: 56%

Matched peptides shown in **bold red**.

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1 MAFAGVLNDA DITAALEACK AADSFNHKTF FAKVGLTSKS ADDVKKAFAI
51 IDQDKSGFIE EDELKFLQN FKAGARALTD GETKTFLKAG DSDGDGKIGV
101 DEFTALVKA
    
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Unformatted sequence string: [109 residues](#) (for pasting into other applications).

Fig. 6: Amino acid sequence alignment of carp parvalbumin

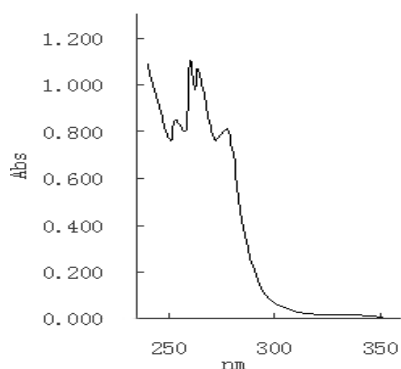


Fig. 7: Ultraviolet absorption spectra of crucian carp PV

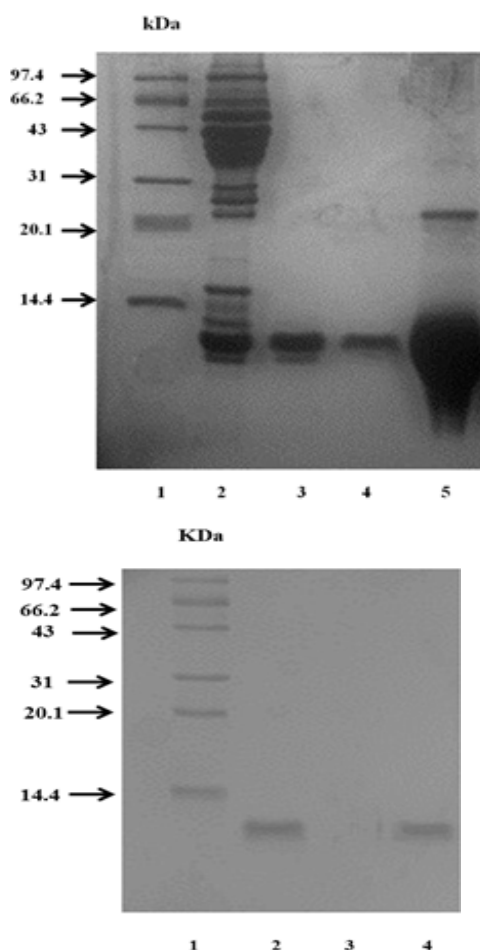


Fig. 8: Graphic for table of contents

Accordingly, ten peptide fragments belonged to one protein. The certainty factor of the six peptide fragments exceeded the threshold in Fig. 6. The protein sequence coverage was 56%, with the matched peptides in bold red (Fig. 7).

Absorption spectra: The absorption spectrum of the high PV purity obtained is illustrated in Fig. 8. *C. carpio* have specific PV absorption peaks in the

260 nm region because of a high phenylalanine to tyrosine ratio and a complete lack of tryptophan. This finding is also consistent with that described in literature (Strehler *et al.*, 1977). The amino acid sequences of fish PV reported in previous studies showed that PV was highly conserved and homologous between the various classes. Furthermore, the sequences were devoid of tyrosine and tryptophan (Hamada *et al.*, 2003; Van Do *et al.*, 2005). Hence, these sequences could not be used to determine the PV content absorption based on the 280 nm wavelength value.

Previous studies reported that PVs in vertebrates have specific absorption peaks in the 260 nm region because of their amino acid sequences. Similar absorption peaks were obtained in this study with UV detection. Thus, the protein showing high purity was PV again.

CONCLUSION

A rapid, simple and efficient method was used to purify the PV allergen. The method involved the use of heating, twice TCA precipitation and gel filtration chromatography. PV was identified with western blot and characterization with MALDI-TOF MS and its characteristic ultraviolet spectrum. Future studies can use our results to further explore PV detection in freshwater fishes.

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REFERENCES

- Aas, K., 1966. Studies of hypersensitivity to fish. A clinical study. *Int. Arch. Allergy Appl. Immunol.*, 29: 346-363.
- Aas, K., 1969. Elsayed, S. Characterization of a major allergen (cod). *J. Allergy*, 44: 333-343.
- Aas, K., 1987. Fish Allergy and the Codfish Allergen Model. In: Brostoff, J. and S.J. Challacombe (Eds.), *Food Allergy and Intolerance*; Baillière-Tindall, London, pp: 356-366.
- Bernhisel-Broadbent, J., S.M. Scanlon and H.A. Sampson, 1992. Fish hypersensitivity. I. In vitro and oral challenge results in fish-allergic patients. *J. Allergy Clin. Immunol.*, 89: 730-737.

- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248-254.
- Bugajska-Schretter, A., A. Pastore, L. Vangelista, H. Rumpold, R. Valenta and S. Spitzauer, 1999. Molecular and immunological characterization of carp parvalbumin, a major fish allergen. *Int. Arch. Allergy Immunol.*, 118: 306-308.
- Coffee, C.J. and R.A. Bradshaw, 1973. Carp muscle calcium-binding protein. I. Characterization of the tryptic peptides and the complete amino acid sequence. *J. Biol. Chem.*, 248: 3305-3312.
- Elsayed, S., 2002. Fish Allergy and the Codfish Allergen Model on Food Allergy and Intolerance. In: Brostoff, J. and S.J. Challacombe (Eds.), 2nd Edn., Saunders, London, pp: 425-433.
- Elsayed, S. and K. Aas, 1971a. Isolation of purified allergens (cod) by isoelectric focusing. *Int. Arch. Allergy Appl. Immunol.*, 40: 428-438.
- Elsayed, S. and K. Aas, 1971b. Characterization of a major allergen (cod): Observation on effect of denaturation on the allergenic activity. *J. Allergy*, 47: 283-291.
- Elsayed, S. and H. Bennich, 1975. The primary structure of allergen M from cod. *Scand. J. Immunol.*, 4: 203-208.
- Elsayed, S. and J. Apold, 1983. Immunochemical analysis of cod fish allergen M: Locations of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *J. Allergy*, 38: 449-459.
- Guo, F., K. Hiroaki and K. Shiomi, 2012. Purification, immunological properties and molecular cloning of two allergenic parvalbumins from the crimson sea bream, *Evynnis japonica*. *Food Chem.*, 132: 835-840.
- Hamada, Y., H. Tanaka and S. Ishizaki, 2003. Purification, reactivity with IgE and cDNA cloning of parvalbumin as the major allergen of mackerels. *Food Chem. Toxicol.*, 41: 1149-1156.
- Hansen, T.K., C. Bindsvlev-Jensen, P.S. Skov and L.K. Poulsen, 1997. Codfish allergy in adults: IgE cross-reactivity among fish species. *Ann. Allerg. Asthma Im.*, 78: 187-194.
- Lindström, C.D.V., T. van Dô, I. Hordvik, C. Endresen and S. Elsayed, 1996. Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of Atlantic Salmon (*Salmo salar*). *Scand. J. Immunol.*, 44: 335-344.
- Pascual, C., M.M. Esteban and J.F. Crespo, 1992. Fish allergy: Evaluation of the importance of cross-reactivity. *J. Pediatr.*, 121: 29-34.
- Sampson, H.A., 1999. Food allergy. Part I: Immunopathogenesis and clinical disorders. *J. Allergy Clin. Immunol.*, 103: 717-728.
- Sampson, H.A., 2003. Food allergy. *J. Allergy Clin. Immunol.*, 111: 540-547.
- Shiomi, K., S. Hayashi, M. Ishikawa, K. Shimakura and Y. Nagashima, 1998. Identification of parvalbumin as an allergen in horse mackerel muscle. *Fisheries Sci.*, 64: 300-304.
- Shiomi, K., Y. Hamada, K. Sekiguchi, K. Shimakura and Y. Nagashima, 1999. Two classes of allergens, parvalbumins and higher molecular weight substances, in Japanese eel and bigeye tuna. *Fisheries Sci.*, 65: 943-948.
- Strehler, E.E., H.M. Eppenberger and C.W. Heizmann, 1977. Isolation and characterization of parvalbumin from chicken leg-muscle. *FEBS Lett.*, 72: 127-133.
- Swoboda, I., A. Bugajska-Schretter, P. Verdino, W. Keller, W.R. Sperr and P. Valent, 2002. Recombinant carp parvalbumin, the major cross-reactive fish allergen: A tool for diagnosis and therapy of fish allergy. *J. Immunol.*, 168: 4576-4584.
- Towbin, H., T. Staehelin and J. Gordon, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *P. Natl. Acad. Sci. USA*, 76: 4350-4354.
- Van Do, T., I. Hordvik and C. Endresen, 2005. Characterization of parvalbumin, the major allergen in Alaska Pollack and comparison with codfish Allergen M. *Mol. Immunol.*, 42: 345-353.
- Zheng, C., X. Wang, Y. Lu and Y. Liu, 2012. Rapid detection of fish major allergen parvalbumin using super paramagnetic nanoparticle-based lateral flow immunoassay. *Food Control*, 26: 446-452.