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Research Article

Inhibitory Effect of Polypeptide from *Argopecten irradias* on Mice with Transplanted H_{22} Hepatocarcinoma and Its Mechanism

[1,2]Yuan Liu, [1]Jian Wang, [2]Yanan Xia, [2]Jianlou Mu, [2]Jianfeng Sun and [2]Jie Wang
[1]Department of Food Science, Hebei North University, Zhangjiakou 075000,
[2]College of Food Science and Technology, Agriculture University of Hebei, Baoding 071000, China

Abstract: The objective of this study was to investigate the inhibitory effect of Polypeptide from *Argopecten Irradias* (PAI) on mice with transplanted H_{22} hepatocarcinoma. PAI was prepared by enzymatic hydrolysis and separation and the results of its structure identification indicated that it contained five polypeptide components with molecular weights of 700-1000 Da. Forty Kunming mice were randomly divided into H_{22} hepatocarcinoma group and low, medium and high dose PAI groups. After ig administration of normal saline and different doses of PAI for 10 days, all mice were inoculated with H_{22} hepatocarcinoma cell suspension to establish models. After continuous gavage for 10 days, the blood was collected from the eyeball and the mice were sacrificed for index detection. It was found that the tumor inhibiting rates of PAI in three dose groups were 28.16, 41.93 and 84.00%, respectively. Compared to H_{22} hepatocarcinoma group, the mutant serum p53, survivin and MDA levels of mice in three PAI-dose groups were significantly reduced ($p<0.01$), the IL-2 and SOD levels were significantly increased ($p<0.01$). The results suggested that PAI had obvious inhibitory effect on mice with H_{22} hepatocarcinoma cells.

Keywords: p53 protein, H_{22} tumor-bearing mice, polypeptide from *Argopecten irradias*, survivin protein, tumor inhibiting rate

INTRODUCTION

Polypeptides from marine organisms have some unique structures and functions compared to those from terrestrial organisms and they have become the hotspot in the fields of new drug development and functional food research. Many marine polypeptides have physiological activities of anti-tumor, anti-oxidation, anti-fungus, anti-virus and immune regulation (Ngo *et al*., 2012; Rameshkumar *et al*., 2009; Aneiros and Garateix, 2004). Polypeptide from *Chlamys farreri* (PCF) is one of the marine bioactive peptides. It can effectively scavenge oxygen free radicals (Zhiwu *et al*., 2006), up-regulate the activity of immune cells and have a protective effect on immune cells damaged by ultraviolet light and ^{60}Co radiation (Wang *et al*., 2003). It also can suppress the oxidative damages in HeLa epithelial cells and fibroblasts, which are caused by UVA and UVB, respectively (Chen *et al*., 2007; Liu *et al*., 2009; Yao and Wang, 2002; Ding and Wang, 2003). Besides, it can significantly reduce the damage effects of UVA and UVB on the skin of Kunming (KM) hairless mouse (Wang *et al*., 2002). However, the reports mostly concerned about PCF extracted from the raw material of *Chlamys farreri*. We seldom see the reports on the obtained polypeptides from other scallops.

Polypeptide from *Argopecten Irradias* (PAI) is a marine polypeptide substance extracted firstly from the *Argopecten irradias* by our research team (Liu *et al*., 2012). Previous studies have shown that PAI had a higher *in vitro* antioxidant activity and radical scavenging ability (Liu *et al*., 2012). Numerous studies have demonstrated that free radicals were closely related with tumor occurrence and development. Free radical levels could be abnormally changed in blood, tumor cells and tumor tissue of the transplanted tumor-bearing animal (Hussain *et al*., 2003; Collins, 2005; Hoelzl *et al*., 2005). In order to elucidate the mechanism of *in vivo* anti-tumor effect of PAI, KM mice were used to establish animal models of transplanted H_{22} hepatocarcinoma in this study.

The objective of this study was to explore the anti-tumor mechanism of PAI by examining the effects of PAI on the tumor inhibiting rate by gavage, pathological morphology and serological marker levels in mice. The study also aimed at laying a theoretical foundation for further development and utilization of PAI.

Corresponding Author: Jie Wang, College of Food Science and Technology, Agriculture University of Hebei, Baoding 071000, China
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MATERIALS AND METHODS

Experimental animals, tumor strains and reagents: *Argopecten irradias* were purchased from Qinhuangdao; acid protease was purchased from Biotopped; Specific Pathogen Free (SPF) grade KM male mice were purchased from Beijing HFK Bio-Technology Co., LTD (animal quality certification number: SCXK (Beijing) 2009-0004). Murine ascites hepatoma H_{22} tumor-bearing mice were purchased from the Fourth Hospital of Hebei Medical University. Survivin, p53, interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), Superoxide Dismutase (SOD) and malondialdehyde (MDA) detection kit were purchased from Beijing Dong Song Biotechnology Co., Ltd.

Preparation and structure identification of polypeptide from *Argopecten irradias*: *Argopecten irradias* were properly washed and their viscera were removed. They were ground, added with water and mixed into homogenate at a certain solid-to-liquid ratio (1:6.25). The pH was adjusted to 3.53. An appropriate amount of acid protease (1312.78 U/g) was added and placed in a constant temperature water bath pot for enzymolysis for 4 h. The enzymes were then inactivated in boiling water for 10 min. After cooling, the suspension was centrifuged at 10000 rpm for 30 min. Supernatant was taken from the mixture and the freeze-dried powder of hydrolysate was obtained after vacuum freeze-drying. Then it was dissolved into ultrapure water to make the solution with the concentration of 1.0 mg/mL, after which the separation and structural identification of it were carried out by RP-HPLC and MALDI TOF/TOF mass spectrometer.

Establishment of animal models: Two murine ascites hepatoma H_{22} tumor-bearing mice were sacrificed by cervical dislocation 10 days after vaccination. After the abdominal skin was disinfected, the milky white as cites was extracted with an empty needle under a sterile condition and was diluted with sterile saline solution into H_{22} hepatoma cell suspension with cell viability of 2×10^7 mL^{-1}. Mice in each group were inoculated subcutaneously with the above suspension at 0. 2 mL/mouse in armpits of the right hind leg.

Grouping and test methods: After adaptively fed under the test conditions for seven days, 40 KM mice were randomly and equally divided into four groups: Model group, low dose group (PAI 500 mg/kg/d), middle dose group (PAI 1000 mg/kg/d) and high dose group (PAI 1500 mg/kg/d). In addition to normal feeding, each mouse in the model group was administered with 0.2 mL/d normal saline by gastric perfusion and mice in the low, middle and high dose groups were administered with PAI by gavage at the doses of 500 mg/kg/d, 1000 mg/kg/d and 1500 mg/kg/d, respectively. Mice in each group were injected with H_{22} hepatoma cell suspension in hind leg armpits on the 11th day to establish subcutaneous transplanted tumor models. Gastric perfusions were then performed continuously for 10 days.

Sample collection: Twenty four hours after the last gastric perfusion, mice were weighed and eyes were removed from each mouse for blood collection. The collected blood was placed at room temperature for 30 min and then centrifuged at 3000 rpm for 20 min at 4°C. Serums were separated and stored at -20°C for serum marker determination. Mice in each group were sacrificed by cervical dislocation after blood collection. Then solid tumors, spleens and thymuses were stripped off and weighed after blood was dried on filter papers. Tumor inhibiting rates and immune organ indexes in mice of each group were calculated based on the following formula:

Tumor inhibiting rate (%) = Average tumor weight in the negative control group (C) –Average tumor weight in the experiment al group (T)/Average tumor weight in the negative control group (C) × 100%

$$\text{Thymus (spleen) index} = \frac{\text{Thymus (spleen) weight}\ (mg)}{\text{Mouse weight}\ (g)} \times 10$$

Effects of PAI on serum immune markers of mice: After the separation of the serums of mice, The ELISA kit was used for detecting marker levels of mutant p53, Survivin, IL-12, Malondialdehyde (MDA) and Superoxide Dismutase (SOD).

Pathological observation of tumor tissues in mice: The tumor tissues were taken and immediately fixed in 10% neutral formalin. Then routine paraffin embedding, sectioning (5 μm), HE staining and resin adhesive mounting were performed. The pathological changes in the tissue sections were observed and photographed under an optical microscope.

Statistical analyses: SAS 9.1 software was used for statistical data processing and analysis. T-test was used to analyze the difference between the two groups. Rank sum test was used in case of variance heterogeneity. The probability level of 5% ($p<0.05$) was considered statistically significant, while that of 1% ($p<0.01$) was considered highly significant.

RESULTS AND DISCUSSION

Preparation and structure identification of PAI: There were five components obtained in PAI after separation by RP-HPLC, which were mixed with equal ratio of matrix solutions (5 mg/mL CHCA, 50% CAN

and 0.1% TFA), respectively. Then 0.8 µL of the mixed sample was taken for structural identification by 4800 Plus MALDI TOF/TOF mass spectrometer (AB SCIEX). The results of the first and second order mass spectrum were showed in Fig. 1 and 2, respectively. The analysis showed that the amino acid sequences of the contained five polypeptide components were found as follows: Cys-Cys-Ser-His-Thr-Arg (Fig. 2A m/z of 706.3) Asn-Gly-Trp-Val-Thr-Arg (Fig. 2B m/z of 718.3), Gly-Asn-Pro-Met-Arg-Arg (Fig. 2C m/z of 730.3), Asp-His-Trp-Lys-Arg (Fig. 2D m/z of 741.4) and Cys-Thr-Tyr-Gly-Pro-Val-Leu-Arg (Fig. 2E m/z of 908.5).

Tumor formation and growth situation in experimental mice: For the model group mice, tumors were formed after 7 days and the tumor growth was rapid. On the other hand, the tumors were formed only

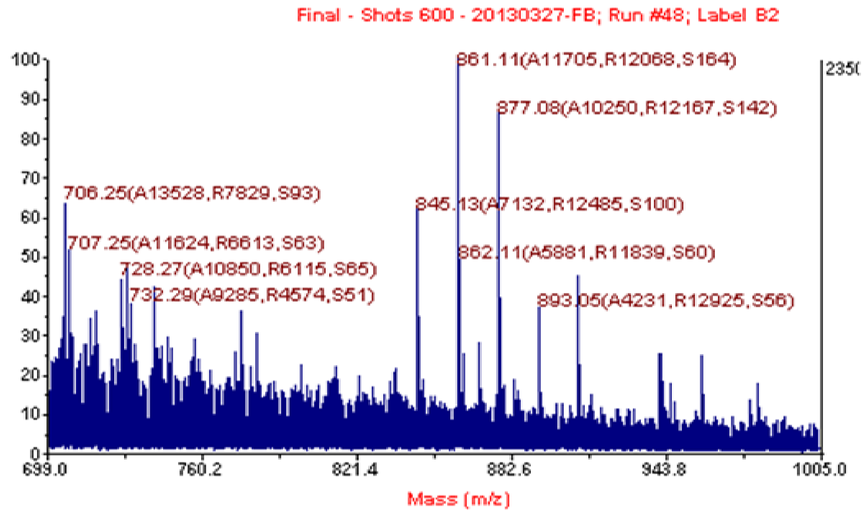
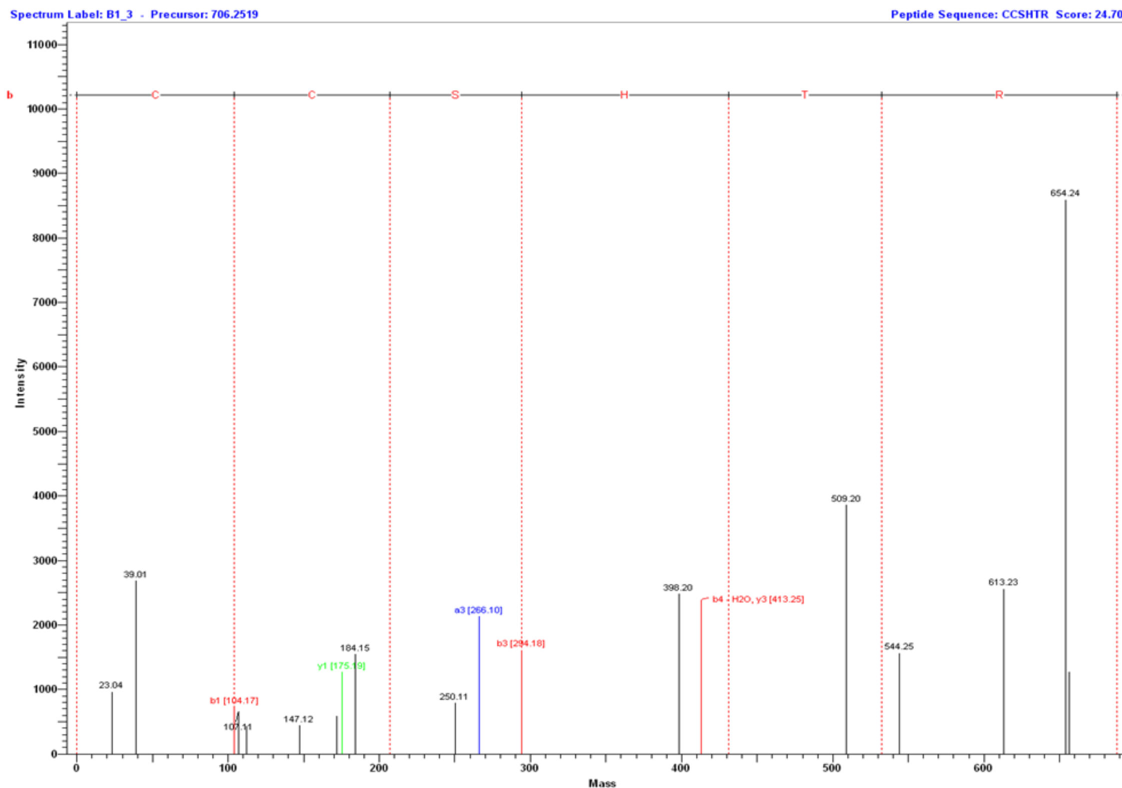
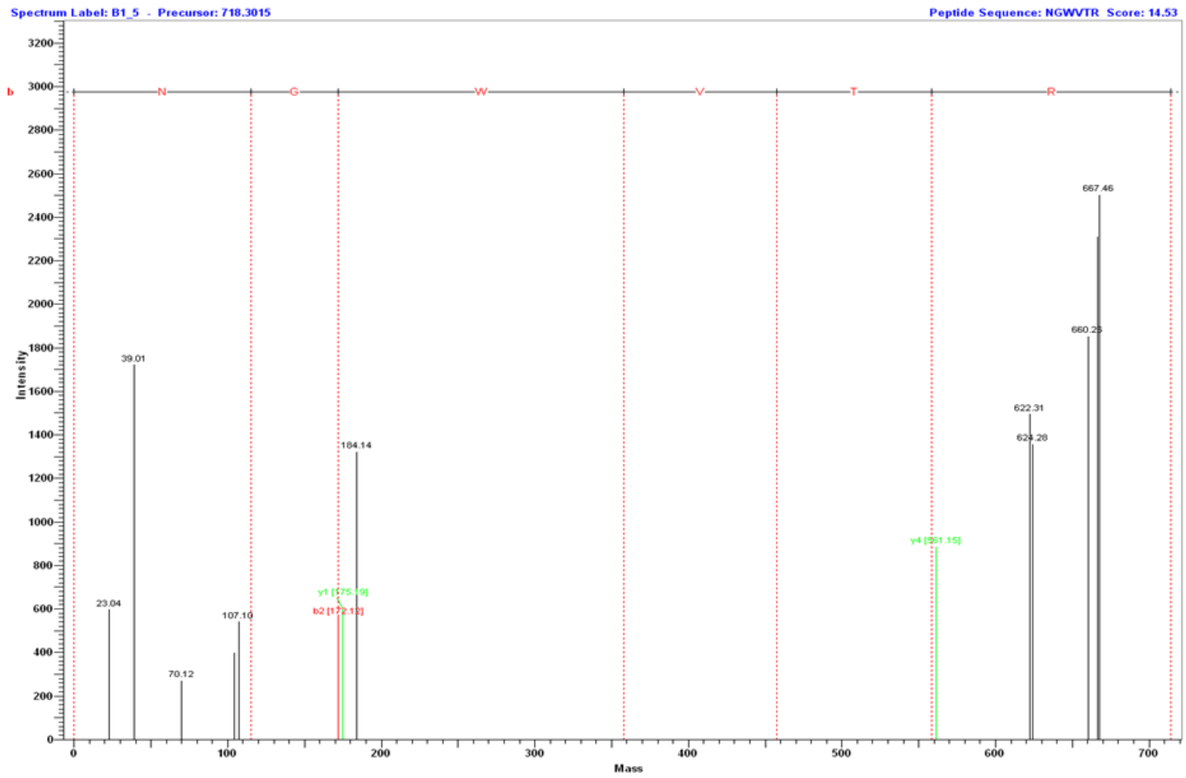


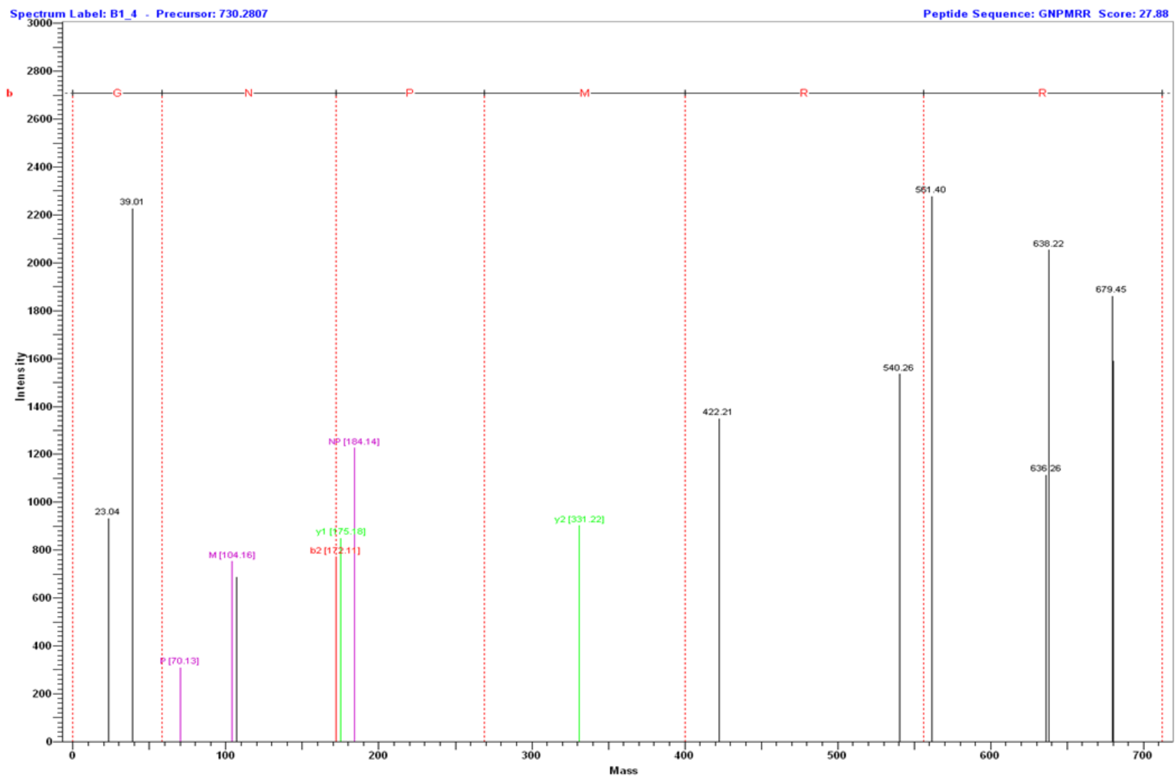
Fig. 1: Mass spectrogram of polypeptide from *Argopecten irradias*



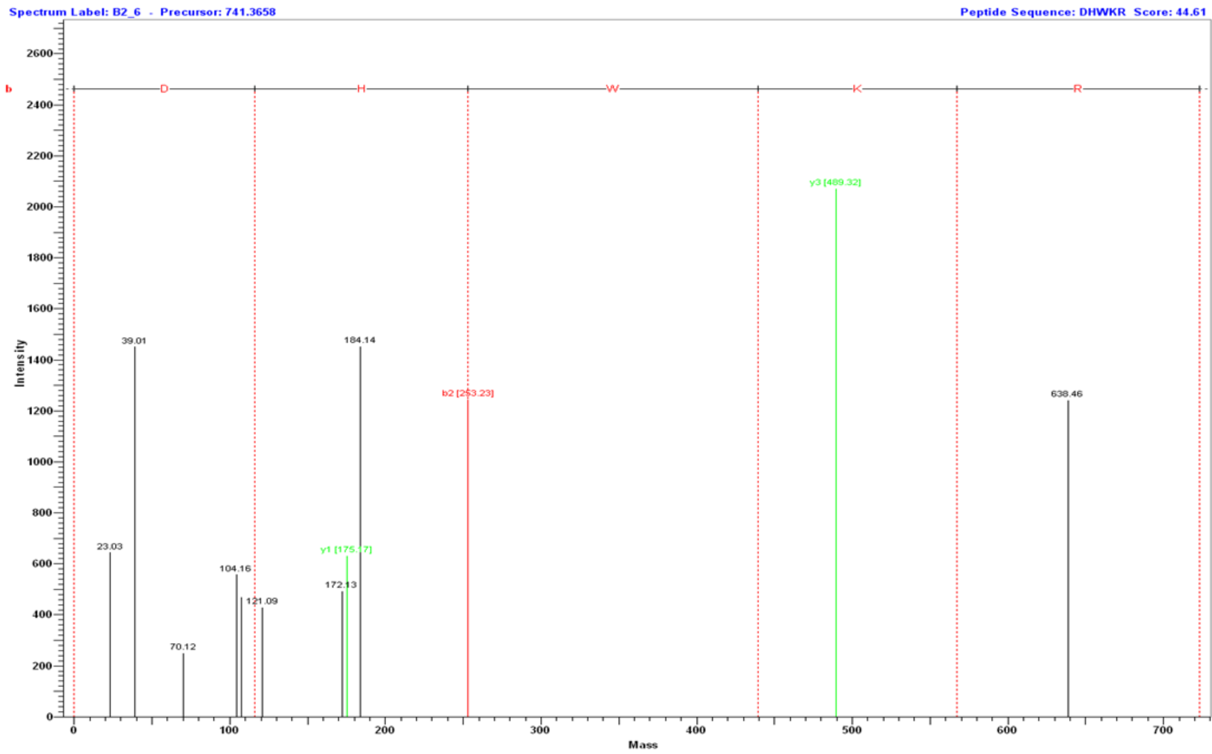
(a) (MS/MS of m/z 706.3)



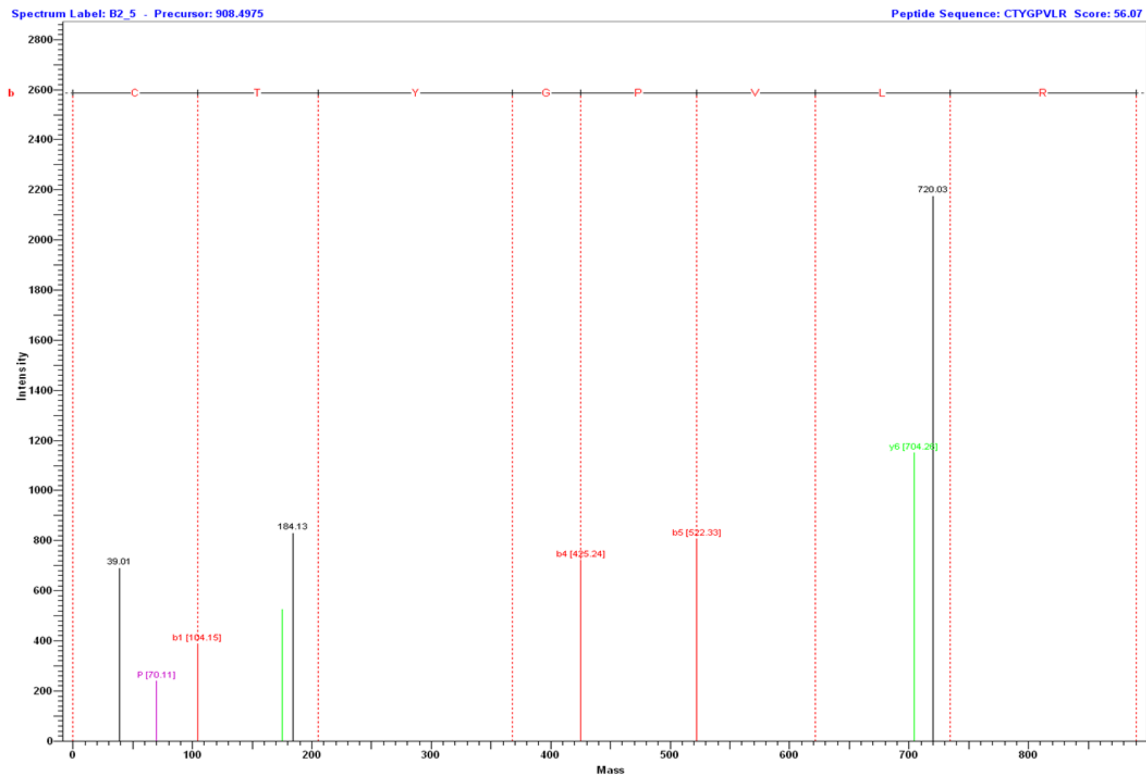
(b) (MS/MS of m/z 718.3)



(c) (MS/MS of m/z 730.3)



(d) (MS/MS of m/z 741.4)



(e) (MS/MS of m/z 908.5)

Fig. 2: MS/MS spectrogram of polypeptide from *Argopecten irradias*

Table 1: Effect of PAI on organs and serum markers of tumor-bearing mice (n = 10, $\bar{X} \mp S$)

Group	Average thymus weight (g)	Average spleen weight (g)	Thymus index (mg/g)	Spleen index (mg/g)	p53 (Mg/mL)
Model	0.09±0.01	0.17±0.02	23.62±4.17	46.18±2.66	92.39±2.72
Low dose	0.10±0.02	0.17±0.02	26.48±3.63	46.58±2.96	79.64±3.92※※
Low P dos	0.10±0.03	0.18±0.04	26.85±3.95	47.09±2.57	75.67±4.26※※
High dose	0.11±0.03※	0.18±0.03	27.88±4.14※	47.61±3.35	70.31±3.36※※
Group	MDA (Nmol/mL)	SOD (U/mL)	IL-2 (Pg/mL)	Survivin (Pg/mL)	
Model	5.19±0.18	106.81±4.77	164.54±10.17	328.16±14.55	
Low dose	4.21±0.10※※	143.90±4.37※※	229.26±12.17※※	250.64±13.40※※	
Low P dos	4.18±0.09※※	145.62±17.88※※	231.24±9.35※※	233.17±12.77※※	
High dose	4.05±0.18※※	151.16±4.81※※	233.46±9.81※※	221.83±16.52※※	

※$p<0.05$ compared to the model group

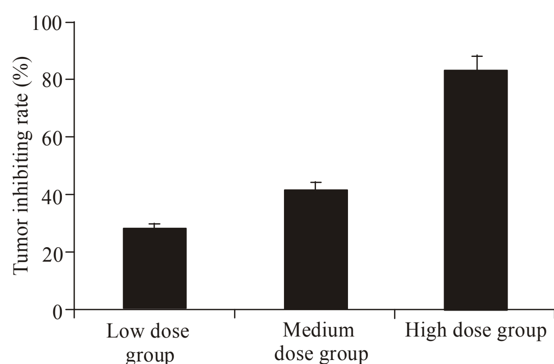


Fig. 3: Effect of PAI on the tumor inhibiting rate in mice

after 9-10 days in low, medium and high PAI dose groups and the tumor growth of them was slower than that of the model group. The tumor formation rate in mice of each group was 100%. The tumor masses of mice in the model group were larger, reaching to 0.54±0.63 g. The H_{22} tumor growths in mice of low, medium and high PAI dose groups were significantly inhibited with the tumor masses of 0.39±0.61, 0.32±0.20 and 0.09±0.11 g, respectively. The difference between the high dose and model groups was statistically significant ($p<0.05$).

Effect of PAI on the tumor inhibiting rate in mice: With increased doses of PAI used in gavage, the tumor inhibiting rates were gradually increased, reaching to 28.16, 41.93 and 84.00%, respectively for low, medium and high dose groups, respectively (Fig. 3).

Effects of PAI on weight and immune organ indexes of mice: The effects of PAI on weight and immune organ indexes are given in Table 1. Thymus weight and thymus index were significantly increased in mice of the high dose group ($p<0.05$) compared to those of the model group. However, there were no significant differences in thymus weight and thymus index in model, low and medium dose groups ($p>0.05$). Spleen weight and spleen index in each group were not significantly different ($p>0.05$).

Effects of PAI on serum markers of mice: The mutant p53, survivin and MDA levels were significantly lower in the serums of mice in all low, medium and high dose groups ($p<0.01$) compared to those in the model group of H_{22} tumor-bearing mice (Table 1). On the other hand, the IL-2 and SOD levels were significantly higher in all above three groups compared to those in the model group ($p<0.01$).

Pathological observation of tumor tissues: After the paraffin-embedded tumor tissue sections were stained by HE, the normal nuclei were dyed with blue-purple and the cytoplasms were dyed with red. Because the nuclear chromatins in cancer cells were increased, the particles were coarsened. The nuclei were hyperchromatic and some looked like ink droplets. Moreover, the nuclei were stained in different depths due to the uneven distribution of nuclear chromatin. More immature cells in tissue caused greater density of DNA, indicating that cell divisions were more vigorous than those in mature cells and the purple blue was deeper. When the tissue growth was abnormal, nuclear accumulation might occur, which would result in partial deep and uneven stains.

Photomicrographs of tumor tissue sections in model group and high dose group were showed in Fig. 4.

Photographs of the tumor tissues in the model group under low and high magnification were illustrated in Fig. 4(A and B), respectively. In Fig. 4A, the blue-purple areas were the vigorously growing tumor cells, in which the white vacuoles were resulted from the dissolution of subcutaneous fat globules. In Fig. 4B, the deep purple blue (nuclear staining) areas were immature and vigorously growing tumor cells. These cells had less cytoplasm and more nuclei and visible binucleate mitotic figures.

Photographs of the tumor tissues in high-dose group under low and high magnification are given in Fig. 4(C and D), respectively. The mitotic figures in tumor cells were decreased, the nuclear aggregation phenomenon was improved and the light color cells indicated the aging cells with small nuclei. The overall performance showed that cells were evenly distributed.

BRUKER ULTRAFLEX-II (4800 Plus MALDI TOF/TOF) was used for structural analysis of the proposed PAI. The product contained five polypeptide components with molecular weights of 700-1000Da and

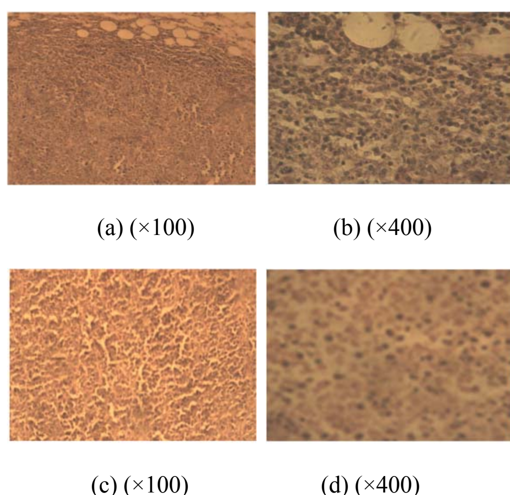


Fig. 4: Photomicrograph of tumor histopathology

their amino acid sequences were determined. There were some differences in molecular weight and amino acid sequence between it and the polypeptide which was isolated from *Chlamys farreri* (4 polypeptide components with molecular weights of 800-1000 Da) by Du *et al*. (2000). The result could possibly due to the difference of scallop species or extraction methods.

The *in vivo* tumor suppression test found that all PAI gavages with doses of 500 mg/kg/d, 1000 mg/kg/d and 1500 mg/kg/d could significantly inhibit the growth of transplanted hepatocarcinoma in mice and the tumor inhibiting rates showed a dose-effect relationship. The histopathological observation of tumor tissues also showed that the obtained PAI had significant anti-tumor biological effects.

Thymus and spleen are important organic immune organs and the observation of their weight changes can visually reflect the changes of immune functions in mice. The results showed that thymus weight was increased in each PAI dose group; especially the high dose group was significantly increased, indicating that PAI had a certain immune enhancement effect on H_{22} tumor-bearing mice.

The p53 gene is a tumor suppressor gene, which is divided into wild-type and mutant. And its products are also divided into wild-type and mutant. Wild-type p53 protein has a trans-activation function and a broad spectrum tumor inhibiting effect. Mutant p53 protein is a tumor-specific marker protein and is associated with the tumor occurrence and development. It is generally believed that mutant p53 over expression is associated with tumor metastasis, recurrence and poor prognosis (Acun *et al*., 2010; Sirak *et al*., 2009; Zhang *et al*., 2011). Survivin is a new member of an apoptosis-inhibiting protein family, also known as a survival factor. It is hardly expressed in normal tissues, while it is selectively expressed in most malignant tumor tissues including liver cancer. Concurrently, it has dual roles in regulating cell mitosis and inhibiting cell apoptosis. It is closely related in occurring, development and prognosis of tumors and has become an important molecular marker as well as a therapeutic target of malignant tumors (Wang and Meng, 2003; Fukuda and Pelus, 2006). The results showed that PAI could inhibite the growth of H_{22} transplanted tumors with significant reduction ($p<0.01$) of mutant p53 and survivin levels. It indicated that PAI could down-regulate the expressions of apoptosis-related proteins such as mutant p53 and survivin to limit tumor invasion and metastasis ability and inhibit the growth of H_{22} transplanted tumors in mice.

Superoxide Dismutase (SOD) is a kind of active substances derived from living organisms, which can eliminate harmful substances produced in the metabolic process of organisms. Malondialdehyde (MDA) is an end product of the lipid peroxidation reaction induced by free radicals, which can induce poisonous cross-linking polymerization of vital macromolecules such as proteins and nucleic acids. Numerous studies confirmed that oxidative stresses in tumor patients were increased through a variety of ways, which could improve the expression levels and activities of antioxidants such as SOD and can obviously inhibit the growth of tumor cells and induce their apoptosis (Liang *et al*., 2011). Our study results revealed that PAI could significantly improve the serum SOD activities of H_{22} tumor-bearing mice and reduce the content of lipid peroxide MDA, indicating that the proposed PAI has strong antioxidant activities, can effectively prevent lipid peroxidation and improve the antioxidant capacity of organism. This could possibly be another mechanism of PAI to inhibit the growth of H_{22} transplanted tumor.

The IL-2 gene is a cytokine with broad biological activities and a growth factor for all T-cell subsets. It can promote the proliferation of activated B cells and is also involved in antibody response, hematopoiesis and tumor surveillance. Therefore, it reflects the cellular immunity ability to some extent. Thus, the reduction of IL-2 is one of important indicators of the impaired immune functions in tumor patients (Jin *et al*., 2012). The results of this study showed that serum IL-2 levels in low, medium and high dose groups were significantly increased compared to that in the model group, indicating that PAI could also play a regulatory role in tumor immune.

CONCLUSION

In summary, the results suggested that PAI is a promising protective substance against hepatoma. Its anti-tumor effect may be realized possibly through the following ways: enhancing the antioxidant capacity of the body, regulating cytokine levels, reducing the expression of tumor markers protein and inhibitor of apoptosis protein and indirectly inducing tumor cell

apoptosis. However, the specific inhibitory mechanisms of the five components of PAI remain to be further studied.

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