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灭活菌苗免疫的南方鲇外周血液细胞免疫指标的变化

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摘要：以嗜水气单胞菌(*Aeromonas hydrophila*)灭活菌苗为免疫原，以平均体重为86.20 g人工饲养的健康南方鲇(*Silurus meridionalis*)为实验对象，通过腹腔注射免疫原(浓度为1.0×10^{9}/mL)免疫，剂量为0.2 mL/尾，并分别在免疫后的第1、2、4、7、14、21、28天对实验鱼尾静脉采血，进行血液生理指标和血液中吞噬细胞活性的测定。结果显示，嗜水气单胞菌灭活菌苗(FKC)可以增加南方鲇外周血液白细胞数量并引起不同种类白细胞组成比例的变化，提高吞噬细胞的吞噬活性。在免疫后第1、2、4、7、14、21、28天，南方鲇外周血中白细胞数量呈明显上升趋势，其中嗜中性粒细胞、单核细胞、淋巴细胞变化趋势更为明显；而且吞噬细胞活性大为提高，吞噬百分比较对照组平均升高了17.66%，吞噬指数比对照组平均升高了1.16，在免疫后第4天吞噬百分比和吞噬指数均达到最高值，吞噬百分比为48.40%，吞噬指数为4.82；免疫后第21天，吞噬百分比和吞噬指数开始下降，但仍高于对照组。由此表明，使用疫苗后，鱼类非特异细胞免疫在初期(尤其是在第2－7天)明显增强，这是鱼用疫苗在免疫早期保护作用的主要机制之一。

关键词：嗜水气单胞菌；疫苗；细胞免疫；南方鲇；血细胞
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嗜水气单胞菌(*Aeromonas hydrophila*)是危害鱼类、两栖类、爬行类和哺乳类动物的一种条件致病菌，该菌分布广泛，普遍存在于土壤及水体表面。20世纪70年代初，它作为一种人类肠道病原菌引起人们的关注。自1989年以来，江苏、上海、浙江、安徽、湖北、湖南、广东、广西、福建、江西等地都有本病例报道，该病原菌涉及面广，传播迅速，发病率和致死率均很高，给人类和养殖业造成了严重危害[1-4]。对该病的控制，国内目前仍主要以抗生素等化学药物为主，但该菌对抗生素具有一定的抗药性，而且使用化学药物会造成残留等不良后果。

目前，已有不少灭活全菌疫苗问世，这些疫苗在不同程度上都可以提高鱼类的抗病性，减少发病率[5-7]，但对疫苗免疫机理的研究多集中于血清抗体浓度和效价的提高等特异性免疫方面[8]，对使用疫苗后鱼体的非特异免疫力的改变尚缺乏足够的了解。迄今，国内未见针对南方鲇的此类报道。

本研究用灭活嗜水气单胞菌菌苗免疫的南方鲇(*Silurus meridionals*)，观察其外周血白细胞数和各种白细胞的组成比例以及吞噬细胞活性的改变，探讨鱼类非特异细胞免疫在鱼类疫苗诱导的免疫保护中的作用，旨为阐明鱼用疫苗在鱼病防治中的作用机理提供理论依据。

1　材料与方法

1.1　实验鱼及饲养条件

本实验室购买寸片南方鲇，饲养于2.5 m×1.0 m×1.2 m的室内实验鱼池，饲养水为经过曝气的自来水，充气泵增氧，每天投喂适量的猪肝；实验前取大小近似的健康实验鱼90尾(平均体重为86.20 g)于规格50 cm×40 cm×40 cm的玻璃水族箱(每箱15尾，共6箱，分为甲、乙两组，甲组60尾，乙组30尾)暂养1周后，开始实验，其中甲组($n=60$)用于安全性实验和统计检测分析；乙组($n=30$)用于免疫指标测定实验，整个实验期间控制水温在(13±1)℃，并做好日常管理。

1.2　免疫原的制备

将嗜水气单胞菌接种在普通琼脂培养基上，28℃恒温摇床(150 r/min)培养24 h后，平板法测定细菌浓度，用灭菌生理盐水(质量分数0.5%)洗下菌落，离心集菌，再用无菌生理盐水稀释至2.5×10^{9}/mL后，加入1%的福尔马林，28℃灭活24 h，即成福尔

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马林灭活的嗜水气单胞菌整体菌苗(formalin-killed *Aeromonas hydrophila* cells,FKC),离心洗涤3次,再用灭菌生理盐水将菌液稀释至1.0×10^8/mL,置4℃冰箱中备用。

1.3 金黄色葡萄球菌悬液的制备

将金黄色葡萄球菌接种于淡水鱼类琼脂培养基(FWA)斜面上,28℃下培养24 h。用无菌生理盐水(质量分数0.5%)洗下菌落,按常规方法制成菌量为2.5×10^8/mL的菌悬液[9]。加入1%的福尔马林,28℃灭活24 h,用无菌生理盐水清洗3次,调整细菌浓度到1.0×10^8/mL,保存于4℃冰箱中备用。

1.4 灭活菌苗的安全性实验

将甲组实验鱼($n=60$)随机分为实验组($n=50$)和对照组($n=10$),其中,实验组胸鳍基部注射按上述方法制得的嗜水气单胞菌灭活全菌苗FKC,注射剂量为0.2 mL/尾,观察7 d。对照组注射等量无菌生理盐水(质量分数0.5%),观察7 d。

1.5 实验鱼的检测与统计分析

分别于安全性实验开始的第1天(即注射FKC的当天)和安全性实验1周后的第1天和第7天随机取注射过FKC的实验鱼6尾,将每次捞取的6尾鱼分为两组(第1组5尾,第2组1尾),每尾均从尾静脉采血0.2 mL,3.8%柠檬酸钠抗凝,分别进行白细胞计数和白细胞分类计数,每一指标测定均重复3次。用SPSS11.0统计软件包(χ^2-检验和单因素方差分析)进行分析比较。

1.6 免疫与采血

将乙组实验鱼分为免疫组和对照组,每组15尾。其中,免疫组每尾从胸鳍基部注射0.2 mL菌浓度为1.0×10^8/mL的FKC,对照组每尾从胸鳍基部注射0.2 mL浓度为0.5%的无菌生理盐水。免疫后,于第1、2、4、7、14、21、28天从各组中随机取1尾鱼,从尾静脉采血0.2 mL,3.8%柠檬酸钠抗凝,将抗凝血液均分为2份,1份用于红、白细胞计数和白细胞分类计数;1份用于吞噬功能实验。每尾鱼在采血前测量其全长、体长和体重(表1),采血后放回池内继续饲养。

表1 对照组和免疫组南方鲇常规生物学指标($n=7$)

Tab.1 Common biological index of southern catfish in control and immunized groups($n=7$)

组别 Group	平均全长/cm Average total length	平均体长/cm Average body length	平均体重/g Average body weight
对照组 Control	24.17	22.09	85.60
免疫组 Immunization	24.89	22.41	86.68

1.7 血液生理指标测定

红细胞和白细胞(RBC和WBC)计数:用章元凯[10]所用A液与B液混合液稀释后,在Neubauer氏计数板上计数。每尾鱼重复计数3次。

白细胞分类计数(differential leucocyte count,DLC)值测定:用Wright氏染液染色的血液涂片,每尾鱼涂片3张,每张在油镜下随机观察100个白细胞,记录其分类百分比,即在观察的100个白细胞中某种白细胞所占的百分比。

1.8 吞噬功能实验

取样分别在实验的第1、2、4、7、14、21、28天进行。每次随机分别从各组捞起1尾鱼,实验重复2次。在上述抗凝血中加入0.05 mL菌浓度为1.0×10^8/mL的金黄色葡萄球菌悬液,充分混匀后于25℃下水浴60 min,水浴期间每隔10分钟摇匀1次,然后以1 000 r/min离心5 min,吸取白细胞层制作涂片。每个血样涂片3张。待片上水分临近全干时滴加甲醇固定5—7 min,蒸馏水冲洗,Wright氏染色15 min。自来水冲洗后,再用蒸馏水冲洗,晾干,油镜观察记录结果。

吞噬活性分别以吞噬百分比(phagocytic percentage,PP)和吞噬指数(phagocytic index,PI)表示并按下式计算:

$$PP=N_{100}/100\times100\%$$

$$PI=N_1/N_2$$

式中,N_{100}指100个吞噬细胞中参与吞噬的细胞数;N_1为被吞噬的细胞总数;N_2为吞噬细胞的细胞数。

2 结果

2.1 安全性实验结果

甲组中注射了FKC的南方鲇经过1周饲养后,

未发现有死亡现象或染病症状。说明所用疫苗的注射剂量对南方鲇是安全的。

2.2　统计分析

χ^2 检验显示，第 1 组鱼各数据间差异不显著。单因素方差分析结果亦表明，第 1 组数据和第 2 组数据间差异均不显著。由此可见，现有样本能很好地代表所属总体且各样本间差异不显著，即从总体中随意抽取 1 尾进行实验均能代表总体。

2.3　外周血液白细胞数及各种白细胞组成的变化

免疫组外周血白细胞数量从注射疫苗后第 2 天起开始升高，第 4－28 天白细胞平均数在 $79.8\times10^3/mm^3$ 至 $84.2\times10^3/mm^3$ 范围内作小幅波动（图 1）；单核细胞和嗜中性粒细胞分类百分比变化趋势相似。单核细胞百分比从注射后第 2 天开始升高，到第 7 天达最高值，其分类百分比为 13.35%，而此时对照组外周血液单核细胞分类百分比为 3.58%，较对照组提高了 3 倍，此后逐渐减低至第 28 天恢复到起始状态；嗜中性粒细胞百分比亦从注射后第 2 天开始上升，第 4 天即达到 9.80%，第 7 天达到最大值 10.06%，而此时对照组外周血液嗜中性粒细胞为 5.99%，较对照组提高了近 2 倍，此后其分类百分比也呈下降趋势，至第 28 天达到最低但仍比对照组略高；单核细胞的增殖幅度明显高于嗜中性粒细胞（图 2 和图 3）。此时免疫鱼的外周血涂片显示单核细胞有大量聚集现象；免疫鱼淋巴细胞在第 2－4 天比对照组低，从第 7 天起开始增殖，第 21 天达到最大值 47.29%（图 4）；在注射了灭活嗜水气单胞菌疫苗的 28 d 中，免疫组的外周血血栓细胞分类百分比始终略低于对照组（图 5）。

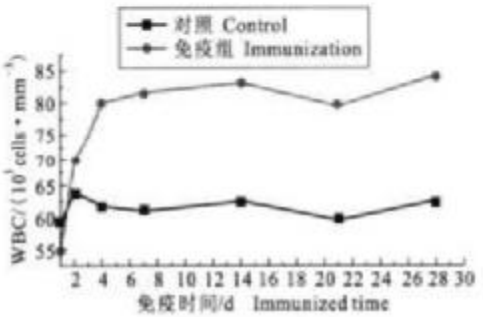

图 1　对照组和免疫组鱼白细胞数的变化

Fig.1　Changes of leukocyte number in blood of control and immunized catfish

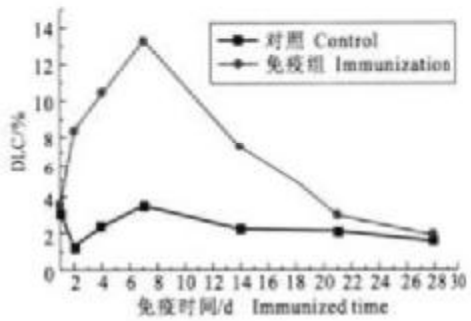

图 2　对照组和免疫组鱼单核细胞分类百分比变化

Fig.2　Kinetics of monocyte percentage in leucocytes of blood in control and immunized catfish

DLC: Differential leucocyte count

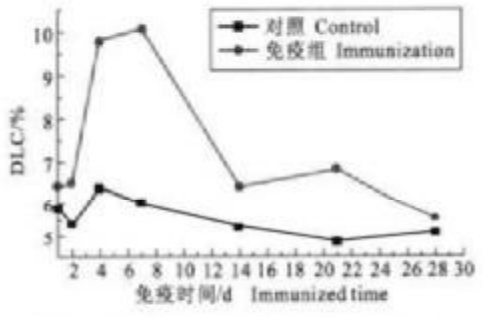

图 3　对照组和免疫组鱼嗜中性粒细胞分类百分比变化

Fig.3　Kinetics of granulocyte percentage in leucocytes of blood in control and immunized catfish

DLC: Differential leucocyte count

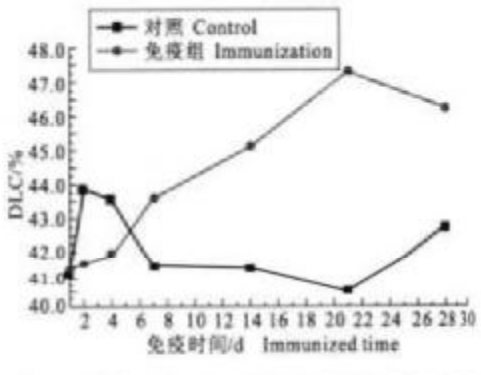

图 4　对照组和免疫组鱼淋巴细胞分类百分比变化

Fig.4　Kinetics of lymphocyte percentage in leucocytes of blood in control and immunized catfish

DLC: Differential leucocyte count

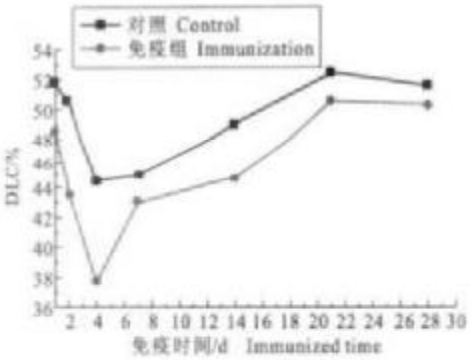

图5 对照组和免疫组鱼血栓细胞分类百分比变化

Fig.5 Kinetics of thrombocyte percentage in leucocytes of blood in control and immunized catfish
DLC: Differential leucocyte count

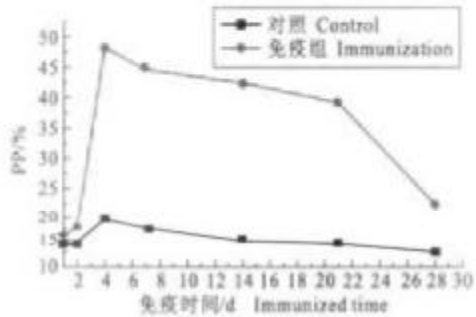

图6 对照组和免疫组鱼吞噬细胞吞噬百分比的变化

Fig.6 Kinetics of phagocytic percentage of phagocytes in blood of control and immunized catfish

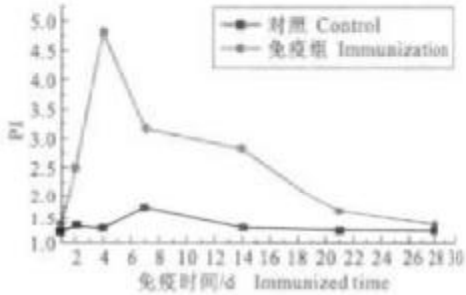

图7 对照组和免疫组鱼吞噬细胞吞噬指数的变化

Fig.7 Kinetics of phagocytic index of phagocytes in blood of control and immunized catfish

3 讨论

Köllner[11]给虹鳟(*Oncorhynchus mykiss*)注射嗜水气单胞菌灭活疫苗后,发现在15℃时,血液和脾中白细胞数量显著增加,血液和脾中白细胞分类比也发生显著变化。在本实验中,给南方鲇注射疫苗后,其外周血单核细胞、嗜中性粒细胞、淋巴细胞分类百分比均明显提高,其中单核细胞从免疫后第2天起即大量增殖,至第7天达到最高并呈现聚集现象,嗜中性粒细胞的变化趋势与单核细胞的相同,这与Köllner实验结果相似。而外周血中淋巴细胞的数量在接受免疫后的头6 d内并没有明显变化,其增殖首先发生在第7天,以后逐渐上升至第21天达最大值。这主要是因为淋巴细胞在接受抗原刺激后,其增殖首先发生在脾脏内,随血液循环而进入外周[11],故其增长趋势较单核细胞和嗜中性粒细胞滞后。由此可以推断,在外周血中,淋巴细胞较单核细胞和嗜中性粒细胞发挥作用迟,即在接受疫苗免疫的最初阶段非特异性的单核细胞及嗜中性粒细胞最先发挥抗菌作用。

2.4 外周血吞噬细胞的吞噬百分比和吞噬指数的变化

注射疫苗后,外周血吞噬细胞的吞噬百分比和吞噬指数都明显增高,吞噬百分比由对照组的12.48%－19.57%上升至免疫组的16.00%－48.40%,吞噬指数由对照组的1.21～1.74上升至免疫组的1.36－4.82。吞噬百分比平均比对照组提高了17.66%。吞噬指数平均比对照组高出1.16。免疫后第4天吞噬百分比和吞噬指数均达到最高值,吞噬百分比为48.40%,吞噬指数为4.82,免疫后第21天,吞噬百分比和吞噬指数开始下降,但仍高于对照组(图6、7)。

研究表明[12-13],鱼类嗜中性粒细胞是体内的吞噬细胞,在某些情况下也许是一种重要的吞噬细胞。鳜鱼(*Karrius bicoarctatus*)机体发生炎症时,其嗜中性粒细胞活跃地向炎症部位迁移并大量吞噬细菌[14];O'Neill[15]在体外条件下观察到虹鳟嗜中性粒细胞吞噬酵母细胞的过程。在本实验中已观察到,在体外条件下,南方鲇的嗜中性粒细胞能吞噬多个金黄色葡萄球菌,且其吞噬百分比和吞噬指数均从注射疫苗后的第2天开始增加,在整个免疫过程中平均分别比对照组提高了17.66%和1.16。这些结果表明南方鲇的嗜中性粒细胞也是吞噬细胞,而且在外源物质进入机体时能快速有效地发挥其吞噬功能。

嗜水气单胞菌是南方鲇出血病的致病菌,其灭活菌苗可显著提高南方鲇血清抗体的凝集效价,抵抗该致病菌的攻击,在接种后第14天血清凝集抗体开始上升,21 d后快速上升,第28天达到峰值,之后缓慢下降[16]。实验中外周血液白细胞数及各种白细胞分类百分比的变化结果表明,从免疫后的第2天起白细胞总数,特别是具有吞噬功能的单核细胞和嗜中性粒细胞的数量均开始增加,其中单核细胞和嗜中性粒细胞在免疫后第7天均达到最大值,分别较对照组提高了3倍和2倍,第14天时亦保持在较高水平;同时吞噬活性实验显示,其吞噬指数和吞噬百分比在第4天时达到最高,第14天以前均保持在较高水平。由此可见,在抗体产生前的14 d内,主要由单核细胞和嗜中性粒细胞大量增生来清除外源物保护机体的。不难看出,非特异细胞免疫在南方鲇抗体形成前及免疫的最初阶段发挥了重要作用。

鱼类是较低等的脊椎动物,它的特异性免疫机制还很不完善,多数鱼类体内只有一种免疫球蛋白IgM,由于缺乏高等动物体液免疫中的主要免疫球蛋白IgG,鱼类不具备抗体生成的二次反应[17-18],非特异细胞免疫在鱼类的免疫防御中发挥重要作用。有不少鱼用疫苗虽然可以产生较强的保护力,但是其血清抗体效价却较低,即受免疫鱼血清凝集抗体滴度的高低与免疫保护效果并不完全一致[19-22],从特异性免疫机制上难以作出合适的解释。研究结果表明,嗜水气单胞菌灭活菌苗诱导受试鱼早期防御能力的提高主要表现在非特异细胞的免疫活性增强方面,从而进一步证明了非特异细胞免疫在鱼病防治上的重要意义。对这种现象的深入研究将有助于全面揭示鱼用疫苗保护作用的机理。

实验中各项指标的测试均采取随机抽取1尾进行重复试验的方法,这要求实验时严格控制实验条件,实验用鱼应在大小和健康状况等方面尽可能一致。由于实验材料为本实验室人工饲养,小池驯养前做过严格筛选,故上述条件较好控制,而且实验前进行的抽样统计分析,结果证明各供实验鱼统计指标间无明显差异,因此实验的随机取样反映了总体,其样本变化趋势代表了总体的变化。

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Changes of cell immunity index in blood of southern catfish, *Silurus meridionalis*, immunized by formalin-killed *Aeromonas hydrophila*

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Abstract: Formalin-killed *Aeromonas hydrophila* cells (FKC) were used as immunogen to immunize healthy southern catfish (*Silurus meridionalis*) which were reared in the laboratory, the average body weight of which was 86.20 g. After the safe-experiment and statistical test, each southern catfish was intraperitoneally injected with 0.2 mL 1.0×10^8 cells/mL formalin-killed *Aeromonas hydrophila*. Then the haematological indices and activity of phagocytes in blood, which was collected from the tail veines of trial catfish, were investigated on the 1st, 2nd, 4th, 7th, 14th, 21st, 28th days after immunization. The experiment showed that an increasing number of leucocytes and the activity of phagocytes as well as a change of percentage of leucocyte populations took place in the blood of southern catfish after the intraperitoneal injection of formalin-killed *Aeromonas hydrophila*. The leucocytes in the blood of trial catfish on the 1 st, 2 nd, 4 th, 7 th, 14 th, 21 st, 28 th days after immunization increased drastically, especially the granulocytes, monocytes, lymphocytes. The dead *Aeromonas hydrophila* could increase the activity of phagocytes drastically. The phagocytic percentage and the phagocytic index in immunized group were 17.66% and 1.16 respectively, higher than those of their counterparts in the control group. The highest activity emerged on the 4 th day after immunization, and the phagocytic percentage was 48.04% and phagocytic index was 4.82. Although it began to decrease from the 21 st day after immunization, the activities of various phagocytes were still higher in immunized group than that in the control group. During the early period, especially from the 2nd day to the 7th day after injection of vaccines, the non-specific cell immunity of southern catfish increased prominently. The present study suggests that enhancing the non-specific cell immunity of fish be an important immune protective mechanism of fish vaccines.

Key words: *Aeromonas hydrophila*; vaccine; cell immunity; *Silurus meridionalis*; blood cell

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cDNA cloning and sequence characterization of an ascorbate peroxidase from marine red alga *Porphyra yezoensis*

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Abstract: Screening of a cDNA library from *Porphyra yezoensis* with probe based upon cytosolic ascorbate peroxidase (APX, EC 1.11.1.11) genes identified a full-length clone apparently encoding a novel type of ascorbate peroxidase. The clone contained an open reading frame for a protein of 242 amino acids, which exhibited sequence similarity to known cytosolic APX from higher plants and contained two motifs conserved in peroxidases. The deduced amino acid sequence of *P. yezoensis* APX showed a hybrid-type structure, which was only found previously in an APX-B from a unicellular red alga *Galdieria partite*, similar to both chloroplastic and cytosolic isoforms of higher plants. It was possible that the hybrid-type structure was an important characteristic of red algal APX. APX cDNA from *P. yezoensis* showed a high (G+C) content (88.4% in the third-base position and 63.7% total) in the coding region. The nucleotide sequence data reported in this paper will appear in the GenBank database under accession number AY282755.

Key words: cDNA; ascorbate peroxidase; *Porphyra yezoensis*; *Galdieria partite*

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In higher plants, the ascorbate-glutathione cycle (ASA-GSH cycle) plays a major role in protection from reactive oxygen species (ROS)[1]. In this cycle, ascorbate peroxidase (APX) catalyzes the reduction of hydrogen peroxide to water by the preferential oxidation of ascorbate[2-3]. Considering the probable role of APX in scavenging ROS, recent studies have focused on the changes in activities of APX in higher plants subjected to several environmental stresses such as ozone, high light, extremes of temperature, salt and paraquat[4-12]. These studies demonstrate that APX message and /or activity is modified by environmental factors, and indicate that APX is critical component that prevents oxidative stress in photosynthetic organisms. Thus, elucidation of the regulation mechanisms of APX in response to environmental stress is a subject of great interest with respect to APX research and will help clarify the oxidative stress-tolerance responses of photosynthetic organisms.

On the basis of enzyme characteristics and amino acid sequences, APXs of higher plants have been divided into at least four distinct cellular compartments: stromal APX and thylakoid membrane-bound APX in chloroplasts, microbody membrane-bound APX, and cytosolic APX[13]. Different APX isoforms differ in amino acid composition and structure, substrate specificity and affinity, and stability during purification[13]. APX activity has been detected in some eukaryotic unicellular algae. In green algae, the subcellular localization and the properties of APX differ from those of higher plants[14]. cDNA clones encoding APXs localized in the stroma of *Chlamydomonas reihardtii* C9 and *Chlamydomonas* sp. W80 were recently isolated[15]. With respect to non-green photosynthetic organisms, only a single gene encoding cytosolic APX has been identified in unicellular red alga *Galdieria partite*[16]. However, in marine macroalgae, none of gene encoding APX has been identified and the role of APX related with antioxidant metabolism is less well understood.

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Porphyra is the most economically important seaweed in the world. *P. yezoensis* has been extensively cultivated particularly in Japan, Korea and China. It is an intertidal marine macroalga which can inhabit a harsh environment where it is subjected to repeated immersion and emersion due to tidal fluctuations. As a result, twice a day, it is exposed to a wide range of environmental stresses including high light, rapid fluctuations in temperature, osmotic stress and desiccation. It is highly probable that *Porphyra* has a higher ability to scavenge active oxygens by the same enzyme system as that found in higher plants and some eukaryotic algae.

As the first step toward understanding antioxidant metabolism in *Porphyra* and elucidating the regulation mechanism of APX in response to environmental stress, we describe the cloning and sequence characterization of a cDNA encoding an APX from *P. yezoensis*. Furthermore, cloning of cDNAs and genes encoding APX of *Porphyra* will facilitate a diverse range of molecular and physiological studies on this enzyme and contribute to breeding on a stress-resistance character of *Porphyra*.

1 Materials and methods

1.1 Algal material and construction of cDNA library

Porphyra yezoensis was collected in Qidong County (Jiangsu Province, China) near the Yellow Sea on Feburary 25, 2003. The live algal samples were transported to the laboratory, washed and then treated immediately or stored at −80 ℃ for experiment.

Total RNA was isolated from *Porphyra* thallus using Trizol Reagent (Life Technologies). Messenger RNA for cDNA synthesis was isolated using PolyATtract mRNA isolation systems (Promega). The cDNA library of *P. yezoensis* thallus was constructed using the ZAP Express cDNA synthesis kit, and Gigapack III Gold packaging extract (Stratagene) by the method that producer provided.

1.2 RT-PCR mediated generation of a homologous probe

Degenerate oligonucleotides were designed based on identical or highly conserved amino acid sequences of known APX genes after an alignment matrix of APX amino acid sequences from different organisms. The sense primer PYAPX1 5'- ATNATGGTNCGNCTNGCNTGG -3' and antisense primer PYAPX2 5'-GCNCCNGANAGNGCNACNAT -3' were used to amplify the partial *P. yezoensis* APX cDNA by PCR. PCR amplification reactions (total volume: 30 μL) were carried out using 4 units of *Taq* DNA polymerase (Takara), 3 μL 10 × *Taq* DNA polymerase buffer, 80 pmol each of primers PYAPX1 and PYAPX2, and about 1 μL cDNA library λDNA template. Thermal cycling parameters were 95 ℃, 5 min, 32 cycles (95 ℃ for 30 s, 50 ℃ for 30 s, 72 ℃ for 45 s), and a final 72 ℃ extension for 5 min. The PCR reaction mixtures were subjected to gel electrophoresis using a 1.5% agarose gel. A 400 bp band was excised, ligated into the pMD18-T vector (Takara) and sequenced. Identity of the products was confirmed by Blast X searches of GenBank. The amplified 386 bp fragment was then randomly labeled with α-^{32}P-dCTP and designated as PAPXc.

1.3 cDNA cloning and genomic Southern hybridization

For cDNA cloning of PY-APX, 85 000 recombinants from the *P. yezoensis* cDNA library were screened using the probe PAPXc. Hybridization was carried out according to reported methods[17].

Seven positive clones were isolated and inserted cDNAs were selected for sequence analysis. Sequence data were submitted to NCBI and searched using the Blast X algorithm and non-redundant amino acid database. Clone PY4 contained almost the complete cytosolic-APX cDNA of *P. yezoensis*. The inferred amino acid sequences were aligned and analyzed with published APX sequences using Clustalx 1.8.

Nuclear DNA Southern hybridization was done with a 350 bp DNA sequence containing partial coding region of PY-APX (from 89 to 438), random prime-labeled with α-^{32}P-dCTP. Hybridization conditions for genomic Southern were the same as described method[17].

2 Results

2.1 Homologous APX probe for library screening

A DNA fragment (PAPXc) of expected size (386 bp) was generated from double stranded *P. yezo-*

sis cDNA by PCR using degenerate primers corresponding to regions conserved universally. DNA sequencing and subsequent Blast X searching for GenBank confirmed that this fragment was amplified from a cytosolic APX cDNA, as evidenced by the higher degree of identity with cytosolic APX sequences of other organisms. The deduced amino acid sequence of PAPXc contained several conserved regions including helix C to F(Fig. 1).

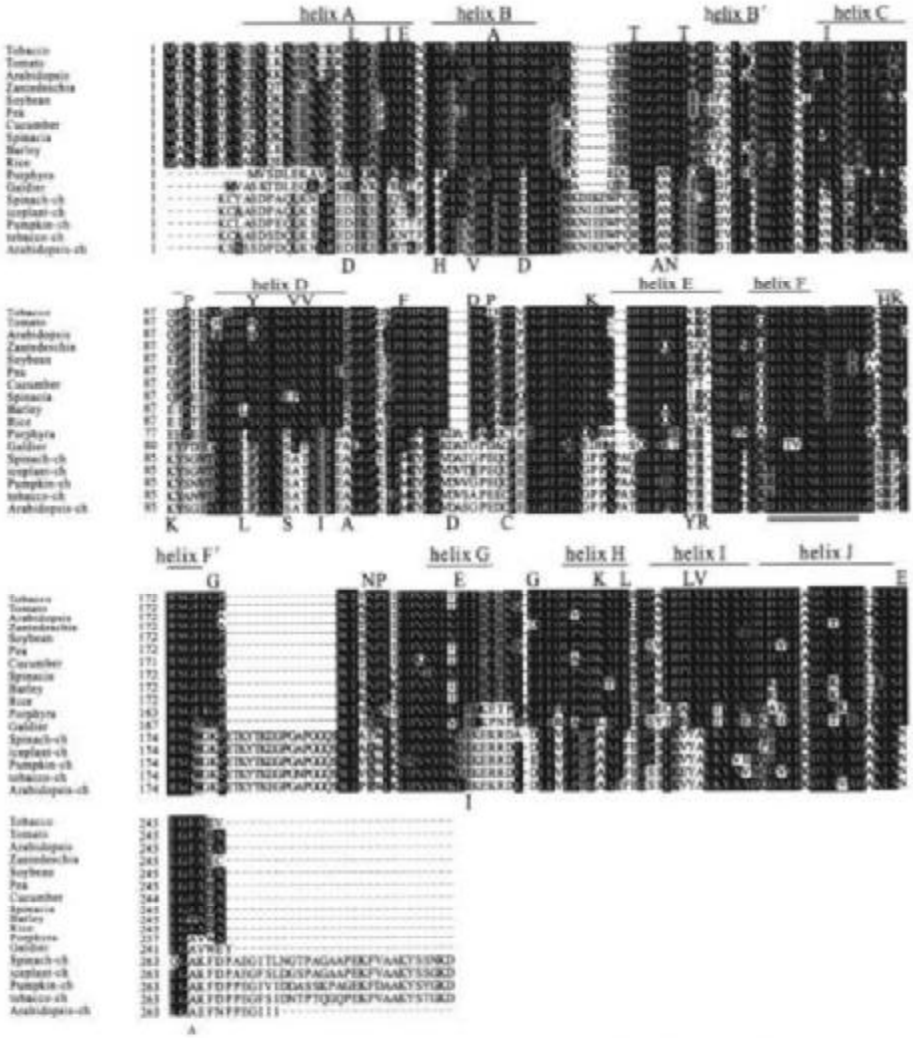

Fig. 1 Comparison of the amino acid sequence of PY-APX with other APXs

Regions from helixes A are aligned. Helixes were assigned according to the structure of cytosolic APX of pea. Black shade indicates identical amino acids, and gray shade indicates similar amino acids. The letters shown under the amino acid sequences indicate residues of PY-APX conserved in the chloroplastic but not in the cytosolic APXs of higher plants. The letters shown on the top of the amino acid sequences indicate residues of PY-APX conserved in the cytosolic but not in the chloroplastic APXs. The peroxidase-active site and the peroxidase-proximal-heme-ligand signatures are underlined and double-underlined, respectively. The proteins shown are cytosolic APXs from tobacco(GenBank accession number BAA12918), tomato (CAB58361), *Arabidopsis* (AAB07880), *Zantedeschia* (AAC08576), soybean (AAI20022), pea (AAA33645), cucumber (BAA13671), *Spinacia* (AAA99518), barley (CAA06996), rice (AAO17000), *Porphyra* (AY273819) and *Galdieria* (AB037537), chloroplastic APXs from *Spinacia* (AB002467), ice plant (AF069316), pumpkin (BAA12029), tobacco (BAA78552) and *Arabidopsis* (X98925).

图 1 条斑紫菜 APX 与细胞质型和叶绿体型 APX 的氨基酸序列比较

从螺旋 A 区域开始比对，螺旋区域定义以豌豆细胞质 APX 为准。黑色区域表示一致的氨基酸，灰色区域表示相似的氨基酸。在序列上方或下方标出的大写字母分别为在细胞质 APX 或叶绿体 APX 中保守，而且在条斑紫菜 APX 中存在氨基酸残基。过氧化物酶的活性位点和过氧化物酶血红素连接信号分别以下划线和双下划线标出。图中比对的序列包括烟草(GenBank 登录号 BAA12918)，番茄(CAB58361)，拟南芥(AAB07880)，马蹄莲(AAC08576)，大豆(AAI20022)，豌豆(AAA33645)，黄瓜(BAA13671)，菠菜(AAA99518)，大麦(CAA06996)，水稻(AAO17000)，紫菜(本文 AY273819)和 *Galdieria* (AB037537)的细胞质 APX 序列，以及菠菜(AB002467)，冰花(AF069316)，南瓜(BAA12029)，烟草(BAA78552)和拟南芥(X98925)的叶绿体 APX 序列。

2.2 Cloning and sequence analysis of PY-APX cDNA

Seven putative APX cDNA clones (PY1-7) were isolated from 85 000 phage recombinants using the homologous APX probe PAPXc. Although these cDNAs were of different lengths due to incomplete cDNA synthesis, all seven clones contained the APX homologous probe sequences. Clone PY4 contained a 1041-bp cDNA insert with an open reading frame of 729 bp (Fig.2). The first ATG located at bases 137-139 was assigned as the start codon. No poly (A) tail or polyadenylation signal was found in this clone, presumably owing to loss of the 3' end of cDNA during the cloning process.

```
1    CCTTGATCCCCCCCCTCGTTCCACCACCACCCCATCGCGGAAACCCGTTCGTCCTACTGCGCCGCATCCCCTTTG   75
76   CCACCGCTGCCTGATCGTGCTGAGTGAGGACGCCCCTTCCCCTCCCTTCCCGCTGCACAAGATGGTGTCTGACCT  150
                                                          M   V   S   D   L
151  GGAGAAGGCGGTCCGCGCCGACTTGCAGGCGCTGATCAAGGAGAAGAACTGCCATGGTATCATGGTCCGGGTGGC  225
      E   K   A   V   R   A   D   L   Q   A   L   I   K   E   K   N   C   H   G   I   M   V   R   V   A
226  GTGGCACGACGCGGGGACCTACTCCAAGGAGGACGGCACCGGCGGCGCCAACGGCACGCAGCGCTTTGCTCCTGA  300
      W   H   D   A   G   T   Y   S   K   E   D   G   T   G   G   A   N   G   T   Q   R   F   A   P   E
301  GAGTGGCCACGGCGCCAACGCCGGGCTAGACATTGCGCGGAACATGTGCGAGGACATCAAGGCCAAGCACCCCGA  375
      S   G   H   G   A   N   A   G   L   D   I   A   R   N   M   C   E   D   I   K   A   K   H   P   E
376  GATCAGCTACGCGGACCTCTACCAGCTCGCCTCGGTTGTGGCGATTGAGGATGCTGGTGGCCCGGTCATCCCTT  450
      I   S   Y   A   D   L   Y   Q   L   A   S   V   V   A   I   E   D   A   G   G   P   V   I   P   F
451  CCGCATGGGCCGCAAGGACGCGGATGCCCCGCAGTGCACGCCCGACGGCCGCCTGCCCGACGCGGACAAGCGCAT  525
      R   M   G   R   K   D   A   D   A   P   Q   C   T   P   D   G   R   L   P   D   A   D   K   R   M
526  GCCCCACCTGCGCGACATCTTTTACCGGATGGGCTTTAATGACGCGGAGATTGTGGCGCTCTCGGGTGCCCACAC  600
      P   H   L   R   D   I   F   Y   R   M   G   F   N   D   A   E   I   V   A   L   S   G   A   H   T
601  GCTCGGCGCCGCCCACAAGGACCGCAGCGGCTTTGATGGCCCGTGGACGAGCAACCCGAACACGTTTGACAACTC  675
      L   G   A   A   H   K   D   R   S   G   F   D   G   P   W   T   S   N   P   N   T   F   D   N   S
676  GTACTTCAAGGAGATTATGAAGGAGACGCCCGAGTCCGGCCTGCTGCATCTGCCGTGGGACAAGGCGCTGTTGGA  750
      Y   F   K   E   I   M   K   E   T   P   E   S   G   L   L   H   L   P   S   D   K   A   L   L   D
751  TGAGCCCGAGTGCAAGGCCCTGGTGGAGACGTACGCGTCGGACCAGGCCAAGTTCTTTGAGGACTACGCCAAGGC  825
      E   P   E   C   K   A   L   V   E   T   Y   A   S   D   Q   A   K   F   F   E   D   Y   A   K   A
826  GCACCAGAAGCTGAGTGAGTTGGGCGCGGTTTGGGCGTGAACCGAGGCTGGCTCGGGTCGCCCCCGGGCGGCTTT  900
      H   Q   K   L   S   E   L   G   A   V   W   A   *
901  GTAGCCGCCACTTGGTTGTTTGATGGCGCAAGGCCTTGCTTGATGGCCTCCTGCATGTGTGTGAGGGCACCATGT  975
976  TGTTGCGATGTGCCAGGCAAGTTTTGTTGTTGGTCTGGTCCTCTTTGTTGGCTTGATGAGTTCTGC          1041
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Fig.2 Nucleotide and deduced amino acid sequences of APX cDNA from *P. yezoensis*

The amino acid sequence is shown in one-letter code below the respective codons and an asterisk indicates a stop codon. Two regions that correspond to the PYAPX1 and PYAPX2 primers are underlined. The position of the nucleotides for cDNA are indicated on both side of the sequences.

图2 条斑紫菜 APX cDNA 的核苷酸及氨基酸序列

氨基酸序列在DNA序列的下面,星号代表终止密码子,下标有横线为两同源简并引物所在处.

APX cDNA from *P. yezoensis* showed a high G + C content. The G + C content of the APX coding region (63.7%) was similar to the average G + C content (65.2%) which was obtained by *Porphyra* EST analysis. One notable feature was that the codons with G or C at the third position were frequently utilized. The G + C content of the third position (88.4%) was much higher than those at the first (63.4%) and second (43.4%) positions.

A preliminary study of genomic organization of APX gene in *P. yezoensis* was determined by Southern blot hybridization of restriction endonuclease-digested genomic DNA, using *Mbo* Ⅰ, which could cut PY-APX probe, *Eco*R Ⅰ, *Hin*d Ⅲ, *Taq* Ⅰ, *Bam* H Ⅰ and *Pvu* Ⅱ which could not. A single band was detected in *Eco*R Ⅰ, *Hin*d Ⅲ, *Taq* Ⅰ, *Bam* H Ⅰ

and Pvu Ⅱ - digested DNA (Fig.3). Our results from Southern blot analysis suggested that one APX gene be present in the *P. yezoensis* genome.

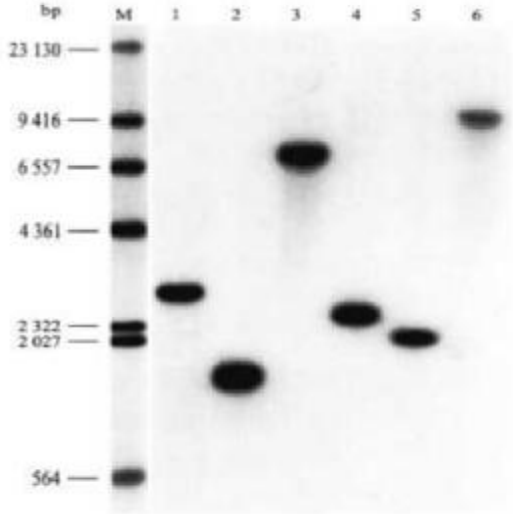

Fig.3 Southern blot analysis of APX gene in *P. yezoensis*. Lane M, λDNA digested with Hind Ⅲ; Lane 1-6, respectively, *P. yezoensis* genomic DNA digested with *Taq* Ⅰ, *Mbo* Ⅰ, *Pvu* Ⅱ, *Hind* Ⅲ, *EcoR* Ⅰ and *Bam* H Ⅰ.

图 3 条斑紫菜 APX 基因 Southern 杂交结果

M. Hind Ⅲ酶切的 λDNA 分子量标记; 1-6: 分别为 *Taq* Ⅰ, *Mbo* Ⅰ, *Pvu* Ⅱ, *Hind* Ⅲ, *EcoR* Ⅰ 和 *Bam* H Ⅰ 酶切后的条斑紫菜基因组 DNA Southern 杂交电泳结果。

2.3 Molecular characteristics of PY-APX

The clone PY4 cDNA represented a protein containing 242 amino acids with a predicted molecular mass of 26 464 D. Comparing the amino acid sequence with the PROSITE database showed two motifs conserved in peroxidases (Fig.1). The first was the peroxidase active-site signature ([SGATV]-x (3)-[LIVMA]-R-[LIVMA]-x-[FW]-H-X-[SAC]) (HGIMVRVAWHDA in PY-APX), except the first residue which was histidine in PY-APX (Fig.1, underlined). The second was the peroxidase proximal heme-ligand signature ([DET]-[LIVMTA]-x (2)-[LIVN]-[LIVMSTAG]-[SAG]-[LIVMSTAG]-H-[STA]-[LIVMFY])(EIVALSGAHTL in PY-APX) (Fig.1, double-underlined). The Arg-28, Asn-61 and Asp-200 residues around the distal His-32 and proximal His-154 residues of PY-APX were conserved in all APX groups as well as the entire plant peroxidase family. These residues were essential for binding of the ligand haem. Trp-170 found in PY-cAPX was conserved in most APX groups and was the third participant in a hydrogen-bonding network together with the proximal His-154 and Asp-200 residues. Levels of amino acid identity between the PY-APX and cytosolic APX in others were 58% similar to the unicellular red alga *Galdieria partita*, and 59% to *Arabidopsis thaliana*.

By a multiple sequence alignment, some residues of PY-APX were found the same as the corresponding residues conserved in the chloroplastic, but not in the cytosolic APX of higher plants (Fig.1, shown by the letters under the amino acid sequences), while some others were the same as those only conserved in the cytosolic APX of higher plants (Fig.1, shown by the letters above the amino acid sequences). The amino-terminal half of PY-APX was richer in chloroplastic residues than carboxyl-terminal half (Fig.1); of 17 chloroplastic residues found in helixes A to J, nine were located in domain I (helix A to D) and only one in domain II (helix F to J). In particular, helixes B and D were similar to those of the chloroplastic isoforms. In contrast, carboxyl-terminal half of PY-APX was similar to the cytosolic isoforms.

3 Discussion

In this report, helixes of other APXs were assigned according to the structure of cytosolic APX of pea[18]. Our results revealed the APX of *P. yezoensis* had a shorter N-terminus than pea cAPX gene for about 10 amino acid residues. The PY-APX had a molecular weight similar to cytosolic APX of higher plants, and lacked Trp-175 residue (corresponded to Phe-166 in PY-APX), which was the major determinant of the greater specificity toward ASA of chloroplastic APX isoenzymes[13]. Furthermore, PY-APX lacked a transit peptide and four additional regions in the internal sequences as found in the chloroplastic isoforms, and also lacked the carboxyl-terminal extension found in stromal, thylakoid-bound, or microbody-bound isoforms of higher plants[13]. These suggested that PY-APX be more likely localized in the cytosol.

Similarly in *Euglena gracilis* and *Galdieria paritta*, APX was found in only in the cytosol[13,15].

However, we found, by a multiple sequence alignment, that amino-terminal half of PY-APX shared sequence similarity with chloroplastic isoforms of higher plants, whereas other parts shared similarity with cytosolic isoforms (Fig.1). Domain I (helix A to D) was richer in amino acid residues conserved in chloroplastic APXs of higher plants rather than cytosolic APXs, while domain II (helix F to J), which formed a pocket at the proximal face of the heme, was similar to the cytosolic isoform. This hybrid-type structure of PY-APX was similar to that of an APX-B localized in the cytosol, which was found in a unicellular red alga *Galdieria partite*[16].

Algal APX isoenzymes had enzymological and immunological properties similar to those of APX isoenzymes from higher plants except for the amino acid sequence of the N-terminus, the affinity for substrates, and/or their stability[15]. Unlike the green algal enzymes, the donor specificity of *Galdieria* APX-B was as high as those of plant chloroplastic APX[14]. Depletion of the electron donor ascorbate caused rapid inactivation of chloroplastic APX of higher plants, while *Galdieria* APX-B was stable under such conditions[14]. Kitajama et al[16] suggested that the specificity of *G. partite* APX-B for electron donors be due to the chloroplastic-like structure of helixes B and D, while its stability under ascorbate-depleted conditions be due to its cytosolic-like domain II. Although none was known about the specificity and stability of PY-APX, the hybrid-type structure of PY-APX suggested that PY-APX have the same enzyme characteristics as those of *Galdieria* APX-B. Because there are only two APX cDNAs (Kitajama et al[16] and this paper) isolated from red algae, it is still an unanswered question whether the hybrid-type structure is an important characteristic of red algal APX.

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