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Nutrients responses of *Pleurotus ostreatus* to slow frozen storage in the short term

Shengjuan Jiang*, Songhua Wang, Yujun Sun and Yuhan Ma

College of Life Science, Anhui Science and Technology University, Anhui Fengyang 233100, China

Contact information for Corresponding Author: Shengjuan Jiang, College of Life Science, Anhui Science and Technology University, Anhui Fengyang 233100, China. Email: jiangsjahstu@126.com

Abstract: To increase the understanding of what was happening with nutrients in *Pleurotus ostreatus* during slow frozen storage in the short term, the metabolite contents of the fruiting body were evaluated. Effects of slow freezing and short-term storage on the variations of nutrients were studied at two temperatures, −20 and −30 °C, at time points during storage. Contents of reducing sugars and vitamin C at all the treatments showed the sustained downward trends. In the pileus, the contents of polysaccharide, proteins, and amino acids increased at first, and then decreased. In the stipe, the contents of all the measured substances maintained a slow decline tendency. The variations of α-amlyase showed a general downward trend with the freezing time. The fluctuation of the activity of POD implied that there was reactive oxygen species production. Significant differences between the freezing/storage at −20 °C and −30 °C were observed in the contents of polysaccharides, reducing sugars, proteins, amino acids and vitamin C. In brief, when 30-min slow freezing had been applied, the contents of most compounds remained the downward tendency or "up-down" trend due to ice damage.

Introduction

Freezing storage is commonly considered to be the least destructive food preservation method. Freezing can thus prevent the growth of micro-organisms, and retain the texture of tissue and nutritional value of the food.[1] In view of these advantages, this method has been implied in the preservation of many biological products, including food,[2] sperms,[3] ovums,[4] embryos,[5] specific proteins,[6] and ectomycorrhizal and arbuscular mycorrhizal fungi.[7]

Freezing mainly influences the state of water. The concentration of the remaining solutes (proteins and carbohydrates) in the cell increases when the water freezes. Then the hyperosmotic stress results in the cell dehydration and shrinkage.[8] As a result, the formation of ice in extra-cellular and intra-cellular compartments will disrupt the homeostasis of the cell system.[9] There are two freezing approaches which are widely applied worldwide, namely slow freezing and fast freezing. The difference between them involves the cooling rate and the speed of ice formation. They have individual advantages in the practical applications. The fast freezing can make the tissues get quickly through the zone of maximum ice crystal formation. Therefore, it is considered as the best freezing method with slight mechanical damages to the cells. The survival rate of day 2-3 cleavage stage embryos is significantly higher after fast freezing compared with slow freezing.[10, 11] However, some researches show that slow freezing from room temperature promotes higher membrane integrity and motility, compared with fast freezing.[12] The human embryonic stem cells have the high recovery rates and a good expansion capacity under the slow-freezing conditions.[13]

The effect of slow freezing on the cryopreservation of human day 3 embryos is better than that of fast freezing by measuring the serum β-HCG levels in pregnancies.[14] So it is illustrated that slow freezing also has its advantages.

Long-term freezing (either fast or slow) is an appropriate way of preserving plant for a long time.[1] Long-term freezing involves extensive exposure of cells to the low temperatures and dehydration.[15] Besides the positive roles, the long-term freezing of foods also has the potential to alter the nutrition and quality, and to cause additional injury.[16] However, the injury and the response of cells to freezing sometimes happen soon after freezing is achieved.[15] The freezing can dramatically reduce the reaction rates of the enzymes in the tissue cells.[17] Many researches have proved that freezing and storage of fruits can significantly change qualitative and quantitative compositions of cells, especially vitamins.[18] And fortunately, many living cells are able to protect themselves against freezing damage by sharp ice crystals by accumulating cryoprotectants such as anti-nucleating proteins and glucose.[17] So it can be deduced that the substances in the cells and tissues fluctuate along with the freezing time.

Pleurotus ostreatus (also called oyster mushroom) is a white-rot fungus belonging to the family of *Pleurotaceae* in the order *Agaricales* within the phylum *Basidiomycota*. It is one of the most widely cultivated edible fungi in the world. The fruit body contains much nutrition, including substantial amounts of proteins, free essential amino acids and an abundance of vitamins, which can be compared to those of eggs, milk, and meat.[19] It also contains approximately 90% water and 10% dry

matter. During the storage, the qualities of *P. ostreatus* decline with losing of the water, opening of the caps, and hardening of the flesh. So it is highly perishable. The addition of the preservatives (such as sodium metabisulphite, citric acid and sulfur dioxide) can prolong the shelf life. However, excess preservatives could ultimately harm human health. So the direct freezing storage without preservatives is the healthy alternative.

Many studies have examined the effects of freezing on fruits and vegetables. In the available literatures, the impacts of freezing and long-term storage on *P. ostreatus* metabolites are mostly studied on amino acids.[20] As far as we know, studies on slow freezing and short-term frozen storage of fresh fruit body are rare. However, the nutritional characteristics can be changed by the storage conditions which influence the chemical compositions.[21] Therefore, to increase the understanding of what is happening with nutraceuticals in slow freezing *P. ostreatus* during the short-term storage, more comprehensive studies are required. The objective of this study was to investigate the initial variations of the nutrients during slow freezing storage of *P. ostreatus* for a short term (30 min).

Experimental

Materials preparation

Fresh *P. ostreatus* mushrooms (5-6 cm in diameter) were collected from the Institute of Edible Fungus (Anhui Science and Technology University) at a mature stage before spore production. And they were processed within 1 h after being harvested. They were sorted, cleaned, washed in cold running sterilized water and

drained. Then the fresh mushrooms were divided carefully into pileus and stipe. Finally each pileus and stipe was cut in half and divided into two groups, individually. The preliminary processing and freezing processes were performed under laboratory conditions.

Freezing procedure

In this work, freezing was performed using regular freezing refrigerator. There was no pretreatment of liquid nitrogen. So the samples were frozen slowly. One hundred gram of pileus and stipe were packaged in food polyethylene membrane bags (thickness: 0.02 mm) and frozen at −20 °C and −30 °C for 30 min, respectively. The frozen mushrooms were taken out of the freezer at 5 min interval. Then the chemical compositions of the frozen mushrooms were immediately analyzed afterward. Six repetitions were performed for each treatment, and each repetition contained 100 g mushrooms.

Analysis methods

Frozen and fresh mushrooms were extracted according to the same procedure. Two grams of tissues were homogenized with 8 mL 0.05 M PBS (pH 7.2) and centrifuged with 10,000 rpm for 15 min at 4 °C. Determination of soluble proteins was carried out by using Bradford Protein Assay Kit (Sangon Biotech Co., Ltd, Shanghai, China) according to the instruction. The results were expressed as g/100g FW (fresh weight).

The polysaccharides were extracted by hot water followed by alcohol sedimentation. In brief, 30 g mushrooms and 120 mL water were added into a flask and extracted at 90 °C for 2 h with stirring. The supernatant was condensed by rotary

evaporation (RE-85Z, Beilun Corporation, China) and removed proteins by chloroform. The anhydrous alcohol was slowly added into the deproteinized solution to a final concentration of 75%. And the mixture was kept for at least 12 h at 4 °C to precipitate the polysaccharides. After the centrifugation, the precipitate was washed and lyophilized to obtain the polysaccharides. The content of polysaccharides was determined by the phenol-sulphuric acid method.[22] The results were expressed as g/100g FW.

The reducing sugars were measured by 3, 5-dinitrosalicylic acid (DNS) method.[23] They were extracted by water at 40 °C and removed the free proteins. The samples were determined spectrophotometrically at 540 nm. The results were expressed as g/1000g FW.

The total amino acids were extracted by PBS (0.05 M, pH 8.0) and determined by ninhydrin methods.[24] The reaction mixture was determined at 570 nm. The results were expressed as g/100g FW.

The vitamin C was extracted by water and determined by phosphomolybdate-blue spectrophotometry.[25] The wavelength was 700 nm and the results were expressed as g/100g FW.

The α-amylase was extracted by the 50 mM Tris-HCl buffer (pH 7.2). After the extraction solution was incubated at 70 °C for 15 min to passivate the β-amylase, the reaction mixture in tubes was incubated at 37 °C for 1 h. The reaction solution contained 10 mg soluble starch and 1 mL enzyme solution to give a final volume of 2 mL. The reaction was stopped by adding 2 mL DNS reagent, followed by incubation

in a boiling water bath for 10 min. After cooling, the absorbance was recorded at 560 nm.[26] One unit of enzyme activity was defined as the amount (mg) of reducing sugar as maltose produced by the enzyme from one gram fresh weight per minute under the standard assay conditions (U/g FW).

The peroxidase (POD) was extracted by PBS (0.05 M, pH 6.5). The activity was measured on the basis of the oxidation of guaiacol using hydrogen peroxide by monitoring an increase in absorbance at 470 nm.[27] One unit of POD was defined as an increase of 0.01 in absorbance per minute under the assay conditions (U/g FW).

Statistics

Statistical analyses of results were done using the one-way analysis of variance (ANOVA) and paired samples *t*-test. Results were graphically presented as the mean ± S.D. of six replicates (n = 6). All the graphs had been prepared using Microsoft Excel 2003. Calculations were done using the SPSS statistics software (Version 18, IBM Corporation, New York, USA) for significance. The differences between the data were tested at a 0.05 significance level (*) or at a 0.01 significance level (**).

Results and discussion

Freezing is a natural way of preserving food. Foods today are flash-frozen. It freezes water in the food into smaller ice crystals, which minimizes the cell structure damage. But slow freezing is commonly used at home. Most home freezers do not freeze foods quickly enough to minimize cell damage in foods. Slow freezing creates large disruptive ice crystals, which damage the cells especially in the beginning of the freezing. Thus nutritional and chemical compositions of mushrooms can be altered

accordingly.[28] In this paper, the metabolic compositions were evaluated and quantified at harvest and after 30 min of frozen storage at −20 °C and −30 °C.

Variations of sugars

The sugars in the cells play very important roles as both nutrients and regulatory molecules in all kinds of life processes.[29] For example, soluble polysaccharide can enhance the activity of immune cells, aid in wound healing and prevent infections.[30] They also can act as the safe antioxidants and anti-tumor agents.[31] As shown in Fig. 1, the contents of polysaccharides in the pileus at −20 °C, and stipe at both −20 °C and −30 °C decreased with the freezing time. At −30 °C, the level of polysaccharide increased dramatically at 5 min ($p<0.01$), and then decreased gradually. By paired samples *t*-test analysis, the contents in pileus at −30 °C were significantly higher than those in the pileus at −20 °C ($p<0.01$). However, the contents in stipe at −30 °C were significantly lower than those in the stipe at −20 °C ($p<0.01$). The fluctuations of polysaccharide in this paper might be related with the freezing stress and the consumption of maintaining the life actions.

The reducing sugar is an important cellular nutrient and is a regulatory metabolite in the cell.[29] After slow freezing, the change trends of reducing sugars of pileus and stipe were similar regardless of the temperatures (Fig. 2). They all fluctuated within a narrow range and showed a descending tendency with the freezing time. The contents in pileus at −20 °C were significantly higher than those in pileus at −30 °C ($p<0.01$). However, the contents in stipe at -30 °C were significantly higher than those in stipe at -20 °C at a 0.01 significance level. The 30-min freezing storage brought about a

reduction in total reducing sugars of approximately 26.3% and 29.6% in frozen pileus at −20 °C and −30 °C, individually. The sugars are the primary substrates of respiration.[32] During the freezing storage, the reducing sugars are consumed by respiration.[33] So the contents of reducing sugars decreased gradually with freezing time in this research.

Variations of proteins and amino acids

Proteins and amino acids are the basic components of all species. Mushrooms contain an abundance of proteins and essential amino acids, which can perform protective roles against tumors [34] and radiation-induced oxidative stress.[35] In this work, the tendency of proteins in both pileus and stipe displayed an approximate bell-shaped curve regardless of the temperatures (Fig. 3). The protein contents of all samples exhibited the peak within 30 min. As far as the protein contents were concerned, the amounts in both pileus and stipe at −30 °C were higher than those at −20 °C ($p<0.05$). Proteins are marginally stable at neutral pH and room temperature. They are liable to change in the frozen states.[36] At the beginning of freezing, the cells are activated to synthesize proteins to maintain the homeostasis or proteins in the cells are relatively concentrated because of the moisture loss.[9] So the protein contents rose at first. With the formation of ice, protein molecules are subjected to chemical and physical changes.[36] Then the denaturation leads to the decrease of proteins.

The effects of short-term freezing on amino acids and proteins were alike (Fig. 4). After the contents of amino acids reached the highest values, they decreased gradually. Furthermore, the pileus and stipe at −30 °C had more amino acids than those at −20

°C. The amount differences of amino acids were significant between the pileus subjected to −20 °C and −30 °C freezing ($p<0.05$). During the storage, the amino acids can be modified by desulphuration, deamination and isomerisation reactions, which can result in a changing of amino acids. The slow freezing and 30-min storage in this assay resulted in the reduction of amino acids ranging from 17.7 to 20.4%. However, fast freezing and 12-month storage brings about a reduction in total endogenous amino acids of 2–16% in frozen *P. ostreatus*.[20] Liu et al has also found that slow freezing, followed by 6 months of storage leads to a significant reduction in free amino acids in *Agaricus bisporus* mushrooms.[28] The comparisons indicate that the loss of amino acids is higher in slow freezing than in fast freezing.

Variations of vitamin C

Vitamin C participates in several physiological processes, including immune stimulation, synthesis of collagen, and iron absorption.[37] The freezing course can influence the amount of vitamin C. As shown in Fig. 5, the freezing (−20 °C and −30 °C) produced the similar dynamics curves and resulted in the gentle declines of vitamin C. The results also showed that the loss of vitamin C influenced by −20 °C was greater than that influenced by −30 °C. Moreover, the differences of vitamin C were very significant between the pileus subjected to −20 °C and −30 °C freezing ($p<0.01$). And the same striking differences were also indicated in stipe subjected to −20 °C and −30 °C ($p<0.01$). Compared with the contents of vitamin C in the fresh material, frozen *P. ostreatus* pileus showed significant decreases within 30 min (38.2% at −20 °C and 28.4% at −30 °C, respectively). Many researches have found

that a loss of vitamin C can not be prevented during storage. Some researches have found that fruits subjected to slow freezing have more vitamin C than fast-frozen fruits.[1] Our results exhibited that the loss at −30 °C was less than that at −20 °C. It implied that the lower temperature of slow freezing would be helpful to the conservation of vitamin C.

Variations of α-amylase and POD

Amylases (EC 3.2.1.1) are basic members of hydrolases widely distributed in all kinds of species, including microbes, plants and animals. They can specifically cleave the O-glycosidic bonds in starch and glycogen.[26] The effects of slow freezing were shown in Fig. 6. The variations of α-amlyase showed a general downward trend with the freezing time. The freezing provided low-temperature condition, which could lower the activity of α-amylase. So the activities of α-amlyase in the two tissues were easily influenced by −30 °C, when compared with −20 °C. The lower activity of α-amylase at −30 °C could reduce the damages of the mushroom. However, there was no significant difference in the two different treatments.

When organisms are exposed to stressful environmental conditions, the production of reactive oxygen species increases and can cause significant damages to the cells.[38] The cells get into a hypo-metabolic state where energy use is both minimized and reprioritized to support crucial vital functions.[8] Freezing increases the cellular susceptibility to oxidative damage owing to its influence on reactive oxygen species (ROS) production.[39] ROS are significant stress factors generated during low temperature storage.[12] POD (EC 1.11.1.7) is a hemo-protein catalyzing the oxidation

by hydrogen peroxide of a number of substrates. It is one of the important members of antioxidant systems. It also plays important roles in many self-defense interactions.[40] When the mushroom countered the short-term freezing, there were no obvious changes of the POD activities in the stipe regardless of treatments (Fig. 7). In the pileus of the two treatments (−20 °C and −30 °C), the activities of POD dramatically dropped at 10 min ($p<0.01$), and increased quickly to the highest values at 20 min. Thereafter, the activities of POD declined rapidly in −20 °C treatment, while in −30 °C treatment such quick decline became attenuated. There was no pronounced difference between the freezing treatments in the same tissue. Overall, variations of POD in pileus showed a roller coaster shape. The fluctuation of POD activity in the pileus implies that there is ROS production and cellular damages induced directly or indirectly by ROS. At the same time, the lower temperature can inhibit the activity of the enzyme. So the POD at −20 °C had the relatively higher activity than that at −30 °C.

Temperature can influence the biochemical reaction process in the organism. The Q10 temperature coefficient is a measure of the change rate in a biological system as a consequence of increasing the temperature by 10 °C. It is a useful way to express the temperature dependence of a process. In this article, −20 °C and −30 °C were chosen to detect the biological variations. As a whole, significant differences between the freezing/storage at −20 °C and −30 °C were observed in the contents of polysaccharides, reducing sugars, proteins, amino acids and vitamin C. The results mean that the variations of those substances can be easily influenced by low

temperature.

In this research, the treatment is similar to the beginning process consumers generate at home when freezing the mushroom. It should be noted that this study has examined only the variations of substances in the first 30-min of slow freezing. Additionally, more species of mushroom samples and the longer storage time should be analyzed, to clearly establish the nutriment profiles of the slow freezing procedure on the mushrooms.

Conclusions

The contents of reducing sugars and vitamin C at all the treatments showed the sustained downward trends. In the pileus, the contents of polysaccharide, proteins, and amino acids increased at first, and then decreased. In the stipe, all the measured substances maintained a slow decline tendency. In brief, when 30-min slow freezing had been applied, the contents of most compounds remained the downward tendency or "up-down" trend due to ice damage.

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Figures

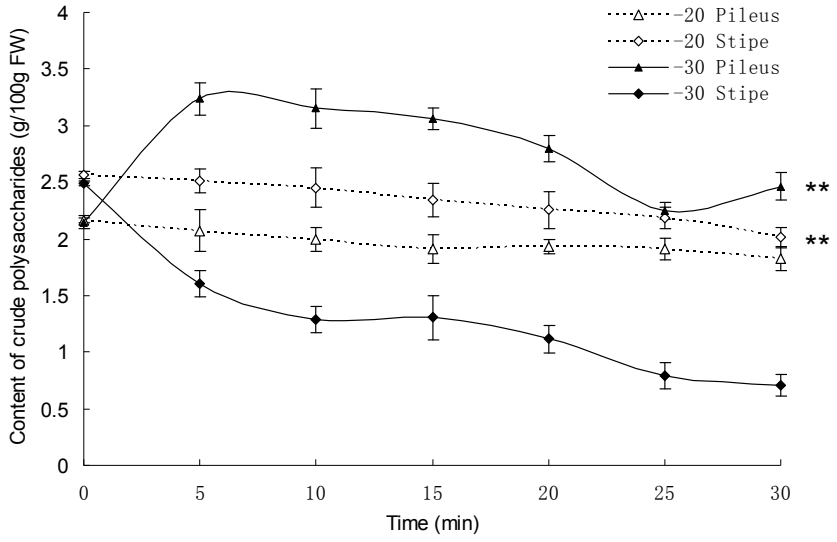


Fig. 1 Contents of polysaccharides at different freezing times and temperatures. The data are marked by mean ± SD (n=6). The data at 0 min are the initial amounts of polysaccharides in the pileus and the stipe of the mushroom before freezing. The symbols (**) mean that the content variations between −20 °C and −30 °C in both pileus and stipe are very significant by paired samples *t*-test analysis at a 0.01 significance level.

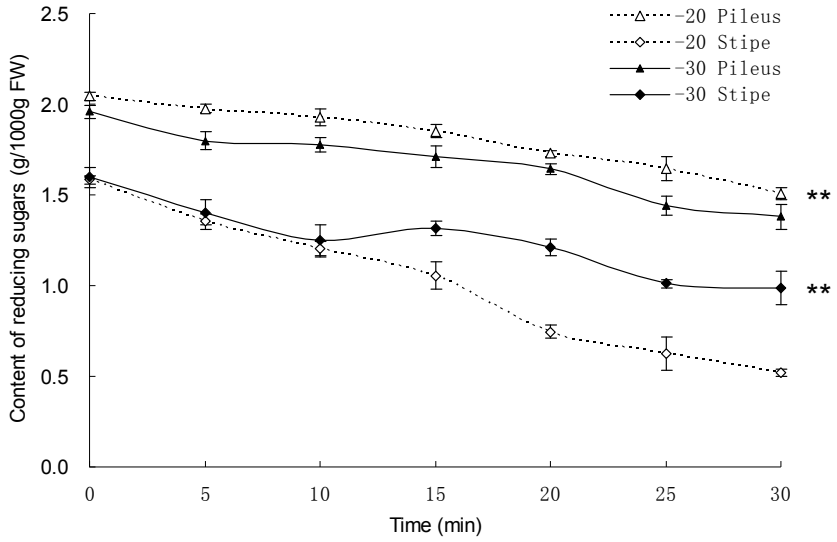


Fig. 2 Contents of reducing sugars at different freezing times and temperatures. The data are marked by mean ± SD (n=6). The data at 0 min are the initial amounts of reducing sugars in the pileus and the stipe of the mushroom before freezing. The symbols (**) mean that the content variations between −20 °C and −30 °C in both pileus and stipe are very significant by paired samples *t*-test analysis at a 0.01 significance level.

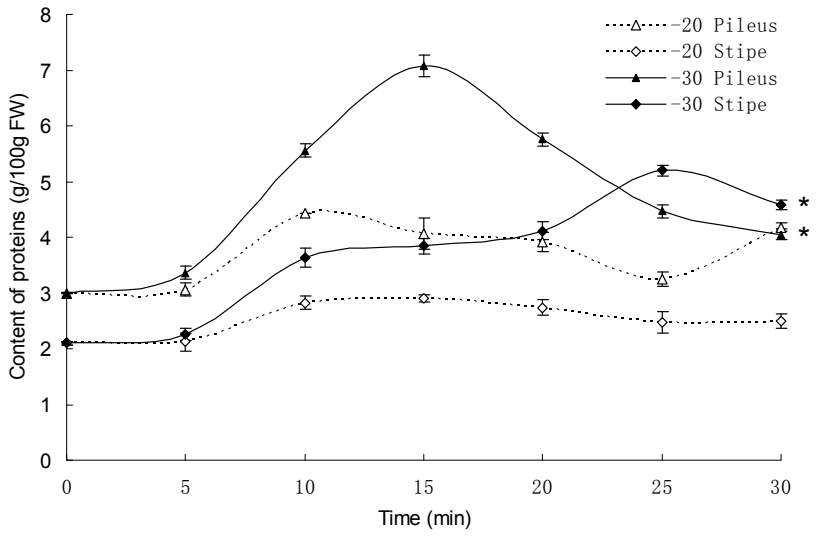


Fig. 3 Contents of proteins at different freezing times and temperatures. The data are

marked by mean ± SD (n=6). The data at 0 min are the initial amounts of proteins in the pileus and the stipe of the mushroom before freezing. The symbols (*) mean that the content variations between −20 °C and −30 °C in pileus are significant by paired samples *t*-test analysis at a 0.05 significance level.

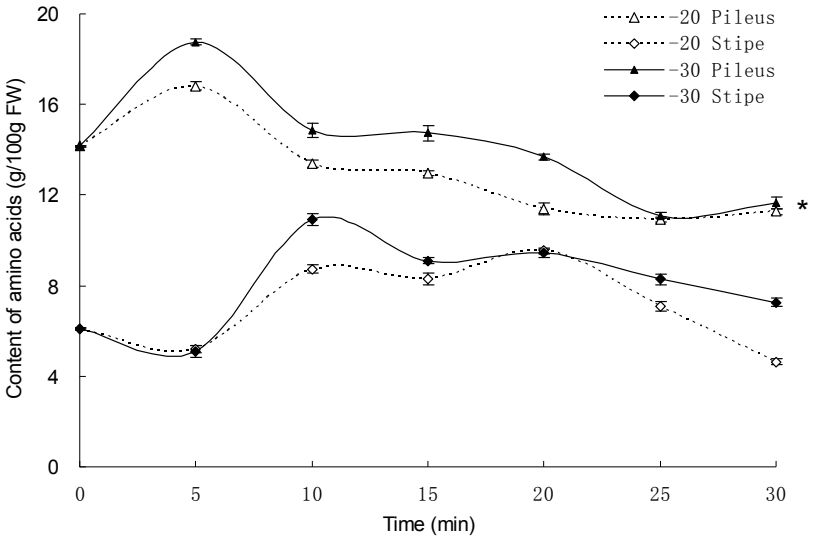


Fig. 4 Contents of amino acids at different freezing times and temperatures. The data are marked by mean ± SD (n=6). The data at 0 min are the initial amounts of amino acids in the pileus and the stipe of the mushroom before freezing. The symbols (*) mean that the content variations between −20 °C and −30 °C in both pileus and stipe are significant by paired samples *t*-test analysis at a 0.05 significance level.

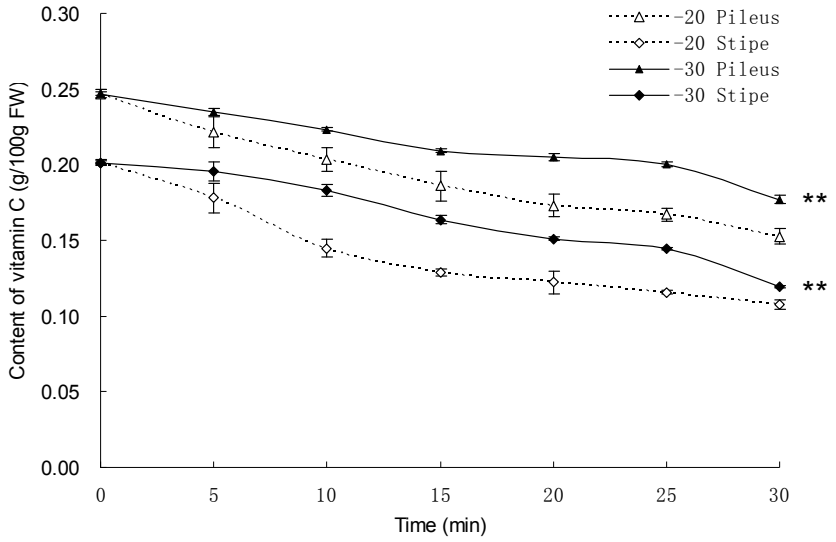


Fig. 5 Contents of vitamin C at different freezing times and temperatures. The data are marked by mean ± SD (n=6). The data at 0 min are the initial amounts of vitamin C in the pileus and the stipe of the mushroom before freezing. The symbols (**) mean that the content variations between −20 °C and −30 °C in both pileus and stipe are very significant by paired samples *t*-test analysis at a 0.01 significance level.

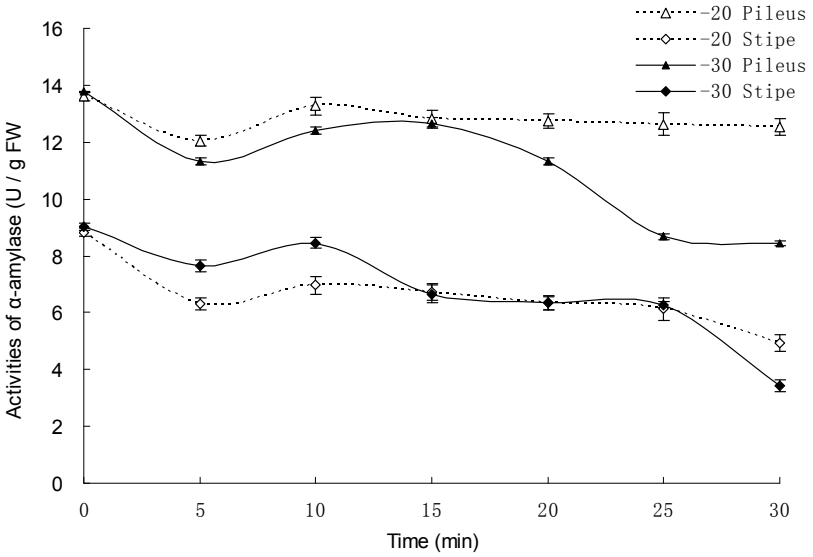


Fig. 6 Activities of α-amylases at different freezing times and temperatures. The data are marked by mean ± SD (n=6). The data at 0 min were the initial activities of

α-amylases in the pileus and the stipe of the mushroom before freezing.

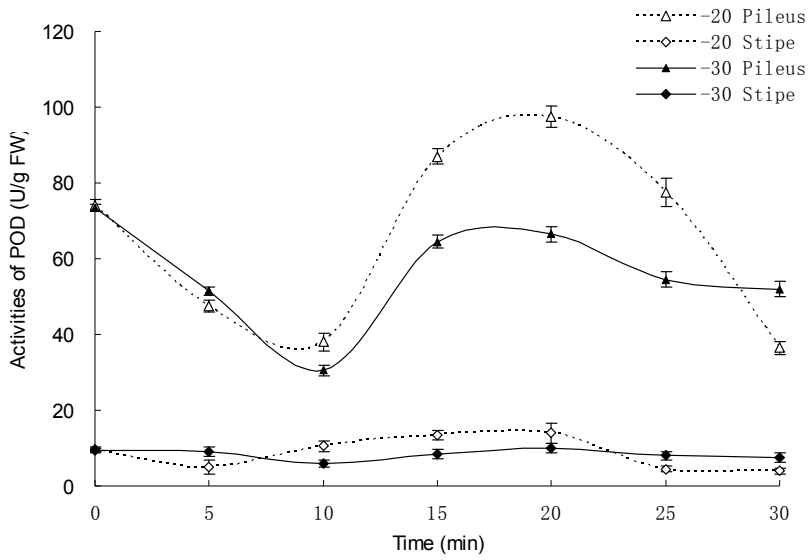


Fig. 7 Activities of POD at different freezing times and temperatures. The data are marked by mean ± SD (n=6). The data at 0 min are the initial activities of POD in the pileus and the stipe of the mushroom before freezing.

Figures

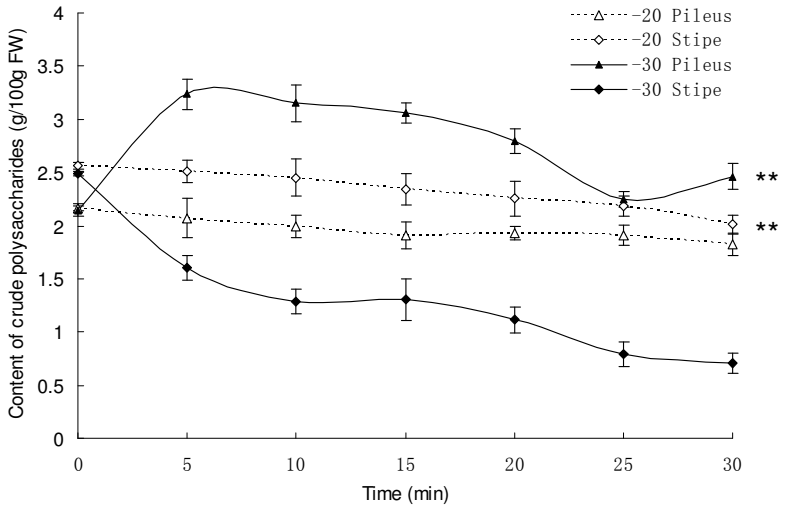


Fig. 1

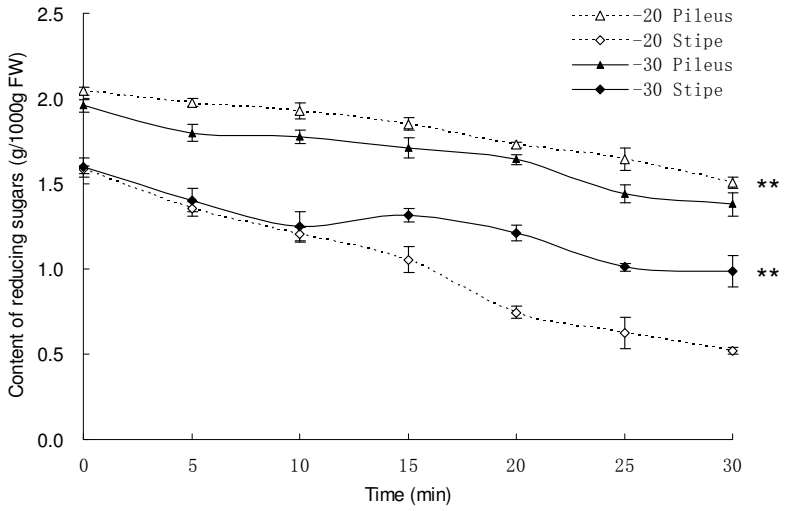


Fig. 2

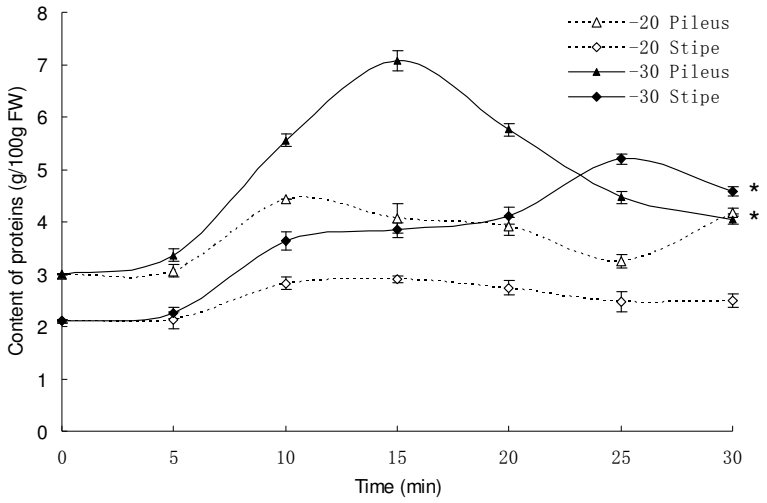


Fig. 3

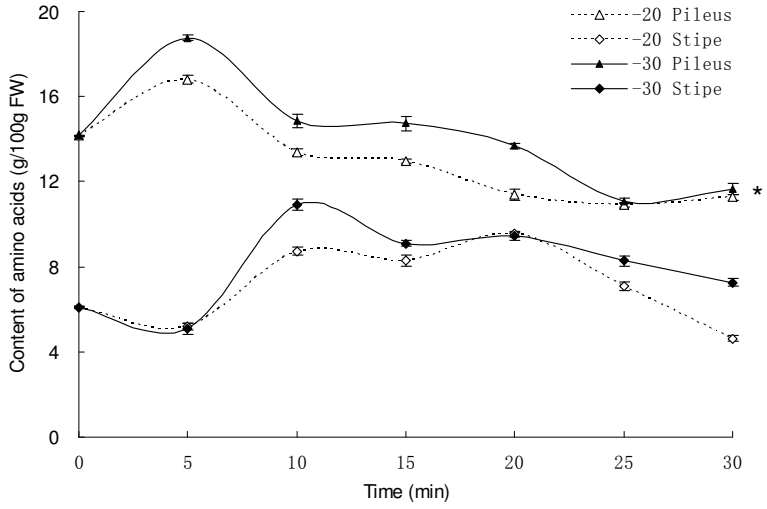


Fig. 4

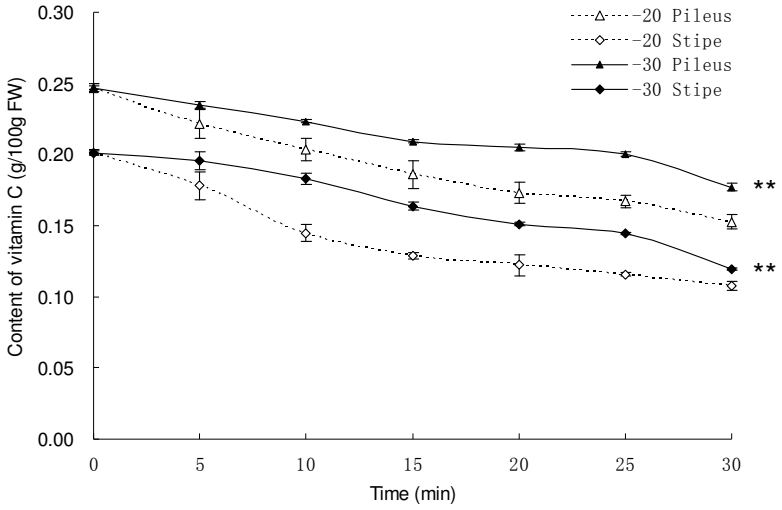


Fig. 5

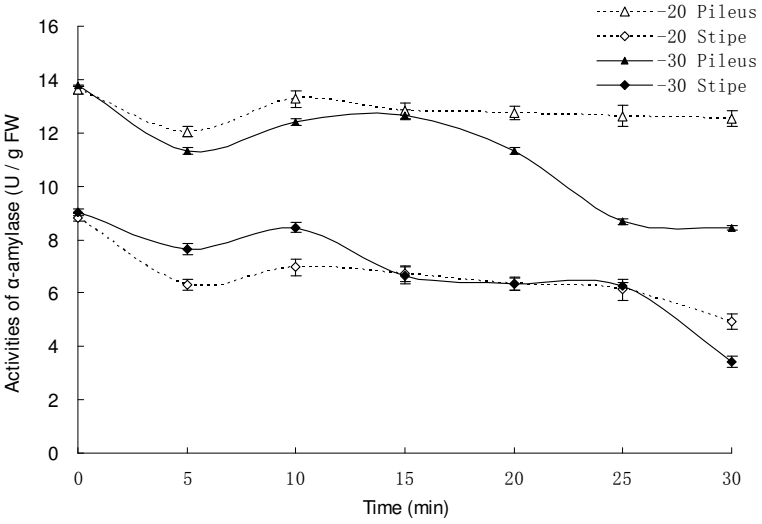


Fig. 6

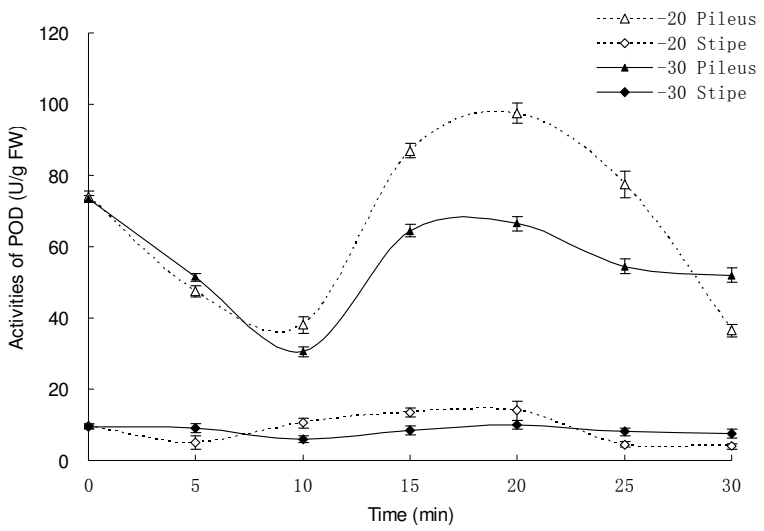
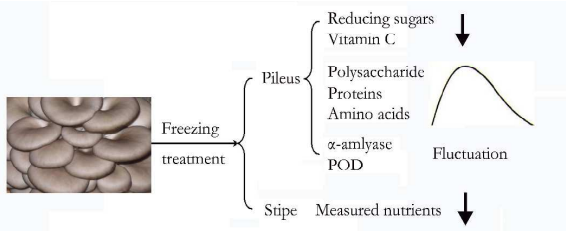


Fig. 7



This paper increases the understanding of what is happening with nutraceuticals in slow freezing *Pleurotus ostreatus* during the short-term storage.