INTRODUCTION

Squid (Todarodes pacificus) mantle muscle contains strong endogenous metalloproteinases that cleave the myosin molecule selectively into heavy meromyosin (HMM) and light meromyosin (LMM). These enzymes were termed ‘myosinas’ by Okamoto et al. because of its high specificity to myosin. Other muscular proteins, such as actin, tropomyosin or troponin, are resistant to this enzymatic action. This enzyme has been reported to be generally distributed in the mantle muscle of other species of squid, but not in cuttlefish.

It is generally believed that high proteolytic activity present in the squid mantle muscle causes problems in the processing and storage of squid meat. For instance, thermal gel of high quality has not been produced from squid mantle muscle due to degradation of myosin, a major protein essential for thermal gel formation during processing.

In a previous paper, we have shown that autolysis is observable at concentrations above 0.3 M NaCl, at which myofibrils dissolve, and that autolysis is suppressed by adding EDTA or pyrophosphate, confirming that the enzyme is a metalloproteinase. Ooizumi et al. have reported that sorbitol suppresses the autolysis of Antarctic krill. The most prominent function of sorbitol established so far is the stabilization of the muscular proteins; surimi from walleye pollack cannot be stored without the addition of sorbitol and/or sucrose because of the unstable nature of its myosin. Another function of sorbitol is in the solubilization of myofibrils. In the present report, we examined whether sorbitol inhibits the autolysis of squid mantle muscle by taking these functions of sorbitol into consideration.

MATERIALS AND METHOD

Live squid (Todarodes pacificus) with a length of 20–25 cm was purchased at a local market in Hakodate. Skinned mantle muscle (10 g) was...
RESULTS AND DISCUSSION

Effect of sorbitol on autolysis at 25°C

We first investigated the effect of sorbitol on autolysis at 25°C. Because autolysis is greatly affected by the presence of NaCl, the effect was studied by varying the NaCl concentration. The homogenate was incubated together with 0 M, 0.5 M or 1 M of sorbitol. The obtained autolysis rates, as measured by the disappearing rate of myosin HC, are shown in Fig. 1a. Autolysis was prominent at approximate concentrations of 0.3–0.4 M NaCl when the medium contained no sorbitol, and the rate was very low not only at concentrations of 0.05–0.1 M but also at concentrations above 0.5 M NaCl. These results were in good agreement with those of previous results obtained. The addition of sorbitol altered the NaCl concentration-dependent autolysis profile, especially at concentrations of around 0.2–0.4 M NaCl. The addition of sorbitol reduced the NaCl concentration required for giving the maximal rate; which is approximately 0.3 M without sorbitol, 0.25 M with 0.5 M of sorbitol, and 0.2 M NaCl with 1 M of sorbitol. The autolysis rate at concentrations above 0.5 M NaCl was reduced upon the addition of sorbitol. The rate at concentrations of 0.05–0.1 M NaCl remained practically unaffected by the addition of sorbitol. These results demonstrated that sorbitol affected the autolysis of squid mantle muscle differently depending on the NaCl concentration.

We have already reported previously that sorbitol promotes the solubilization of carp myofibrils.

Solubilization of myofibrils was studied by measuring the decrease in turbidity of the homogenate with absorption at 350 nm, as described elsewhere. During this experiment, washed homogenate with 0.1 M NaCl–20 mM Tris-HCl (pH 7.5) instead of homogenate itself was used because of the high turbidity of the homogenate resulting from unidentified turbid components present in the homogenate. These turbid components were easily removed after repeated washing.

![Fig. 1](https://example.com/fig1.png) Effect of sorbitol on NaCl concentration-dependent autolysis and solubility of squid mantle muscle. (a) Squid homogenate with (○) 0 M, (□) 0.5 M, and (△) 1 M of sorbitol was incubated at 25°C for up to 5 h. The first-order disappearing rate constant of myosin heavy chain resulting from unidentified turbid components present in the homogenate. These turbid components were easily removed after repeated washing.
rils, and that autolysis can be observed for myofibrils in a dissolved state. Therefore, we studied how these two are related to each other in the aforementioned complicated effect of sorbitol on autolysis. First, we confirmed the solubilization of squid myofibrils by sorbitol (Fig. 1b). Upon the addition of sorbitol, a reduction in turbidity as a result of dissolving myofibrils occurred at a lower NaCl concentration range. The minimal level of turbidity was achieved at about 0.4 M NaCl in the absence of sorbitol, but was at 0.3 M NaCl with 0.5 M of sorbitol, and 0.2 M NaCl with 1 M of sorbitol. These NaCl concentrations required for complete solubilization of myofibrils were very similar to those concentrations required for giving the maximal rate for autolysis (Fig. 1a). Because the protease responsible for autolysis selectively cleaves myosin into HMM and LMM, and because this HMM–LMM junction is protected from proteolytic attack by forming filaments, it is reasonable to suggest that the sorbitol exposes the HMM–LMM cleavage site to the enzyme by dissolving the myofibrils, leading to the enhancement of autolysis. As the autolysis rate at NaCl concentrations above 0.5 M was reduced upon the addition of sorbitol, it is certain that sorbitol clearly has a suppressive effect on autolysis. In other words, suppression was observed only when myofibrils were dissolved. The enhancement of autolysis at around 0.2 M NaCl indicated that the solubilization of myofibrils with sorbitol, which accompanied the exposure of the cleavage site, was much greater than the suppression of the activity by sorbitol. As the autolysis rate at around 0.1 M NaCl was affected slightly by sorbitol, sorbitol was unable to dissolve myofibrils under these conditions. The turbidity decrease of the myofibril suspension at around 0.1 M NaCl upon addition of sorbitol, as shown in Fig. 1b, was suggested to be because of the transparency effect of sorbitol on the myofibril. We noticed that the maximal autolysis rate increased in the presence of sorbitol; 1.7×10^{-5}/s without sorbitol, 2.25×10^{-5}/s with 1 M sorbitol. The enzyme activity seemed optimal at a NaCl concentration much lower than 0.25 M.

We also studied the promotive and suppressive effects of sorbitol on autolysis. The homogenate was incubated at either 0.25 M or 0.5 M NaCl in the presence of various concentrations of sorbitol. Figure 2 shows the results. At 0.5 M NaCl, the autolysis rate without sorbitol was 1.0×10^{-5}/s, and this rate was continuously reduced with increasing sorbitol concentration. The rate with 1 M sorbitol was 5.8×10^{-6}/s, which is roughly a reduction by half. The rate at 0.25 M NaCl without sorbitol, 1.08×10^{-5}/s, was coincidentally similar to that at 0.5 M NaCl; however, the rate increased with increasing sorbitol concentrations up to 0.7 M, at which the maximal rate of 2.72×10^{-5}/s, an enhancement of approximately 2.5-fold, was obtained. At concentrations above 0.7 M sorbitol, the rate decreased. This decrease might be due to the suppression of autolysis by sorbitol achieved at concentrations above 0.5 M. As the myofibrils were fully dissolved upon the addition of 0.7 M sorbitol with 0.25 M NaCl, no acceleration would be expected at sorbitol concentrations above 0.7 M; in turn, the suppressive effect of sorbitol on autolysis that can be observed with dissolved myofibrils became obvious at these concentrations.

Suppressive effect of glycerol and maltitol on autolysis

We found that sorbitol suppressed the autolysis in a concentration-dependent manner once myofibrils were dissolved. We then studied whether other related compounds are also capable of inhibiting autolysis. Tested compounds were glycerol, because half its structure is identical to that of the sorbitol molecule, and maltitol, which has a conjugated structure of two sorbitol units. The NaCl concentration used was 0.5 M NaCl. The disappearance rates of myosin HC in the presence of other...
medium used was 0.5 M, and the temperatures were 25 °C, 30 °C, 35 °C, and 40 °C. Incubation was conducted with or without 1 M sorbitol. Disappearing profiles of myosin HC are shown in Fig. 4. The suppressive effect of sorbitol on autolysis at 25 °C was confirmed (Fig. 4a). At 30 °C, the disappearing profiles of HC with and without sorbitol were almost the same, exhibiting no suppressive effect (Fig. 4b). When we examined the profiles carefully by comparing them with those at 25 °C, we found that, in the presence of sorbitol, the rate increased by raising the temperature to 30 °C, whereas that without sorbitol decreased markedly when the temperature was raised. In general, enzyme reaction rates are greater at higher temperatures, except in cases of thermal inactivation of the enzyme. It is suggested that the enzyme in the medium without sorbitol was partially inactivated at 30 °C. The decreasing profiles of HC at 35 °C were completely different from those at 30 °C (Fig. 4c). When the incubation medium contained no sorbitol, myosin HC decreased slightly during the early phase, but this decrease appeared to stop within 1 h. Probably all of the enzymes involved in the autolysis of squid muscle might have been inactivated by incubation at 35 °C for a short time period when the medium contained no sorbitol. Conversely, myosin HC progressively decreased in the presence of sorbitol, as observed at 30 °C, possibly demonstrating that the enzyme is still active under these conditions. Sorbitol seemed to protect the enzyme from thermal inactivation. When the incubation temperature was raised to 40 °C, practically no HC decrease took place in the absence of sorbitol, and complete inactivation of the enzyme was indicated. In the presence of sorbitol, a slight

these compounds are presented in Fig. 3. Autolysis was suppressed by the three compounds but to differing degrees when their effects were compared in terms of molar concentration. Maltitol had the strongest suppressive effect, whereas inhibition by glycerol was weakest. A reduction in the rate upon the addition of 1 M of each compound was 25% with glycerol, 40% by sorbitol, and 80% by maltitol (Fig. 3a). However, the suppressive effect by these three compounds was almost the same when compared in terms of their percent concentrations; all compounds at reduced the rate by half at approximately 18% (w/v) (Fig. 3b). Although the mechanism was unclear, it is certain that sorbitol suppresses at least one of the following steps in the hydrolysis process of myosin HC: binding of myosin to the enzyme, cleavage of the peptide bond, release of the product. It is possible that increased viscosity of the incubation medium due to high the concentration of compounds suppressed the thermal movement of the enzyme leading to the reaction being inhibited. Another possibility is that the stabilized structure of myosin with sorbitol became resistant to the proteolytic action.

**Effect of sorbitol on the autolysis at higher temperature**

At 25 °C, autolysis in the high-salt medium was suppressed upon the addition of sorbitol in a concentration-dependent manner once myofibrils were dissolved. We next investigated this suppressive effect by varying the incubation temperature. The NaCl concentration for the incubation medium used was 0.5 M, and the temperatures were 25 °C, 30 °C, 35 °C, and 40 °C. Incubation was conducted with or without 1 M sorbitol. Disappearing profiles of myosin HC are shown in Fig. 4. The suppressive effect of sorbitol on autolysis at 25 °C was confirmed (Fig. 4a). At 30 °C, the disappearing profiles of HC with and without sorbitol were almost the same, exhibiting no suppressive effect (Fig. 4b). When we examined the profiles carefully by comparing them with those at 25 °C, we found that, in the presence of sorbitol, the rate increased by raising the temperature to 30 °C, whereas that without sorbitol decreased markedly when the temperature was raised. In general, enzyme reaction rates are greater at higher temperatures, except in cases of thermal inactivation of the enzyme. It is suggested that the enzyme in the medium without sorbitol was partially inactivated at 30 °C. The decreasing profiles of HC at 35 °C were completely different from those at 30 °C (Fig. 4c). When the incubation medium contained no sorbitol, myosin HC decreased slightly during the early phase, but this decrease appeared to stop within 1 h. Probably all of the enzymes involved in the autolysis of squid muscle might have been inactivated by incubation at 35 °C for a short time period when the medium contained no sorbitol. Conversely, myosin HC progressively decreased in the presence of sorbitol, as observed at 30 °C, possibly demonstrating that the enzyme is still active under these conditions. Sorbitol seemed to protect the enzyme from thermal inactivation. When the incubation temperature was raised to 40 °C, practically no HC decrease took place in the absence of sorbitol, and complete inactivation of the enzyme was indicated. In the presence of sorbitol, a slight
decrease in HC content during the early phase (Fig. 4d) was observed similar to that seen at 35°C without sorbitol, indicating that 1 M sorbitol was unable to protect the enzyme from inactivation at 40°C. The effect of sorbitol on autolysis was thus dependent on incubation temperature; that is, suppression at 25°C, and promotion at 35°C. Sorbitol is basically a suppressor of autolysis, whereas under specific conditions in which inactivation of the enzyme might happen, sorbitol functions as a stabilizer of the enzyme, leading to the promotion of autolysis.

Thermal inactivation of the proteolytic enzyme

To confirm the aforementioned suggestion that sorbitol suppresses the thermal inactivation of the enzyme, we studied how enzyme inactivation proceeded at 35°C in the absence or presence of sorbitol. For analysis within the homogenate, we used the following method. Homogenate with or without 1 M of sorbitol was incubated at 35°C for various time periods, as shown in Fig. 4c. The homogenate was immediately transferred to 25°C and incubated for another 5 h. It is possible that if the enzyme activity is lost by preheating at 35°C, then no decrease in HC content by further incubation at 25°C might occur. Myosin HC content before and after incubation at 25°C for 5 h was measured to monitor inactivation at 35°C (Fig. 5). Upon incubation of the homogenate at 35°C, HC content decreased slightly in the absence of sorbitol, as shown in Fig. 4c. In the control homogenate, HC decreased to approximately 65% after incubation at 25°C for 5 h, which was in good agreement with the results shown in Fig. 4a. However, this decrease in HC became less and less with increased incubation period at 35°C, clearly indicating the rapid inactivation of proteolytic enzyme in the absence of sorbitol. Conversely, when 1 M of sorbitol was present in the medium, HC disappeared progressively with increased incubation period at 35°C, which also confirmed the results of Fig. 4c. The content of HC also decreased steadily as the period of incubation at 25°C increased, indicating that the enzyme had not been inactivated by heating at 35°C in the presence of 1 M of sorbitol. The remaining proteolytic activity was calculated from the HC content before and after incubation at 25°C of the pre-incubated homogenate. Inactivation rate was calculated by assuming that the decrease in HC content is the first order reaction, as analyzed in Fig. 4. The inactivation profiles of the enzyme at 35°C with and without sorbitol are shown in Fig. 6. A very quick inactivation of the enzyme was demonstrated in the absence of sorbitol, whereby half of the enzyme’s inactivation was achieved within 10 min. However, proteolytic activity was well maintained up to 5 h when 1 M of sorbitol was added. Heating for 5 h reduced the activity only to 90%. Hence, it was concluded that progressive autolysis at 35°C in the presence of sorbitol was due to the activity of the enzyme stabilized by sorbitol. The proteolytic enzyme present in the mantle muscle of squid is
that the proteolytic enzyme is just as unstable as myosin. Heating for a short period at 35°C or 40°C is one possible way of inactivating the proteolytic enzyme; however, the procedure is always accompanied by myosin denaturation.

It was concluded that sorbitol basically has the ability to suppress the proteolytic activity responsible for the autolysis of squid mantle muscle. However, sorbitol sometimes enhances autolysis under specific conditions, such as those in which sorbitol dissolves myosin filaments and in which sorbitol stabilizes enzyme. Thus, the effect of sorbitol on autolysis should be discussed by considering these three factors. Because autolysis is negligible at concentrations of approximately 0.1 M NaCl, storage of squid mantle muscle without the addition of salt is the best way to prevent autolysis. When squid meat is salted, the addition of pyrophosphate as a chelating reagent to remove the enzyme activator and the addition of sorbitol to suppress enzyme activity might be a way of reducing the effect of autolysis by the enzyme.

Fig. 5 Thermal inactivation of proteolytic activity upon heating at 35°C. Homogenate dissolved in 0.5 M NaCl was incubated at 35°C for various periods in: (a) the absence or (b) presence of 1 M sorbitol, and the incubated homogenate was further incubated at 25°C for another 5 h. Myosin heavy chain content (○) before and (●) after incubation at 25°C was estimated.

Fig. 6 Stabilization of proteolytic enzyme upon addition of sorbitol. Remaining proteolytic activity upon heating at 35°C in either the (○) absence or (●) presence of 1 M of sorbitol was estimated from the extent of disappeared myosin heavy chain by further incubation of the heated homogenate at 25°C, as shown in Fig. 4.

rather unstable if no stabilizing reagents are present. Although the data are not presented, incubation of the homogenate at 35°C for 1 h inactivates myosin ATPase completely, indicating

REFERENCES