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Detection of Giant Myofibrillar Proteins Connectin and Nebulin in Fish Meat by Electrophoresis in 3–5% Gradient Sodium Dodecyl Sulfate Polyacrylamide Slab Gels

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An improved method was investigated for sodium dodecyl sulfate polyacrylamide slab gel electrophoresis (SDS−PAGE) to facilitate the analysis of the giant myofibrillar proteins, connectin and nebulin, in fish meat by using jack mackerel *(Trachurus japonicus)* as the sample fish. It was established that separation of the α-connectin band from the β-connectin band by SDS−PAGE could be achieved by using 3−5% gradient gels with glycerol to facilitate the formation of a gradient with polymerization at 35 °C. SDS−PAGE samples of white dorsal muscle from the jack mackerel were homogenized with a 2% SDS solution containing an inhibitor mixture (1 μg/mL of phenylmethanesulfonyl fluoride, 1 μg/mL of leupeptin, and 1 μg/mL of E-64) and heated at 50 °C for 20 min. Heating these samples at 100 °C for 2 min resulted in the disintegration of connectin but did not affect nebulin. A purified myofibril sample and a whole muscle sample showed similar changes in the overall rate of degradation of whole connectin and nebulin during the postmortem storage period, but it was clear that β-connectin was cleaved from α-connectin during the preparation of myofibrils at the early stage postmortem. Storage of the SDS−PAGE samples at −85 °C was preferable to storage at −18 °C for a long period.

KEYWORDS: α-Connectin; β-connectin; nebulin; SDS−PAGE; jack mackerel

INTRODUCTION

Connectin is an elastic protein of very high molecular weight that was first isolated from rabbit muscle by Maruyama et al. (*1*) and later named titin by Wang et al. (*2*). It consists of doublet components, α-connectin (also called titin 1) and β-connectin (also called titin 2), which is a degradation product of α-connectin. Wang et al. (*2*) also observed a third extremely large polypeptide, referred as to "band 3", by SDS−PAGE analysis of vertebrate skeletal muscle myofibrils that was subsequently named nebulin (*3*). Connectin and nebulin have also been characterized in fish muscle by using SDS−PAGE (*4*−*6*). The role that these extremely large proteins may play in the development of meat tenderness has recently been closely examined. It has been suggested that these two proteins are likely to influence meat tenderness (*7*) because of their unique size, structural properties, and positions in the myofibrils. This might also be the case with fish muscle.

There have been no reports concerning the relationship between the changes of these proteins in fish muscle and textural changes postmortem. Since each of these proteins has an extremely large molecular mass and the masses of α-connectin and β-connectin in fish muscle are very similar, 2400 and 2100 kDa, respectively (*8*), highly porous disc gels (about 2%) have been used for SDS−PAGE analysis of these proteins. However, it is difficult to handle such a highly porous disc gel because of its fluidity. In addition, disc gel is not suitable for western blotting, which is a useful method to identify protein bands separated by SDS−PAGE. The slab gel is more suitable than the disc gel to compare many protein bands from different samples. Tatsumi and Hattori (*9*) have suggested an improved method using 2% slab gel strengthened by agarose, but this method requires complicated temperature control to polymerize the gel, and the gel is easily cracked when the slot-former is removed. On the other hand, α- and β-connectin in beef muscle could be clearly separated by using 5% polyacrylamide gel (*10*) and 3−12% gradient polyacrylamide gel (*11*). We therefore examined the conditions required to separate α- and β-connectin in fish muscle by using normal polyacrylamide slab gel and to obtain reproducible SDS−PAGE patterns of fish connectin.

The results of this study are important for improving technical methods in the field of fisheries science in relation to the quality assessment of fish meat during postmortem storage.

MATERIALS AND METHODS

Samples. Live jack mackerel (*Trachurus japonicus*) were obtained from the local city market and freshly killed by cutting off the hindbrain. Each fish was individually vacuum-packed and stored at 4 °C for a designated time up to 7 days. Only the white dorsal muscle of the fish

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was used. Beef muscle, for comparison with the fish muscle, was obtained from round meat just after slaughter and stored under the same conditions for up to 14 days.

Preparation of SDS−PAGE Samples of the Myofibrils. Myofibrils were purified at 4 °C according to the method of Goll et al. (*12*), as modified by Huff-Lonergan et al. (*13*). The protein concentration was determined by the biuret method as modified by Robson et al. (*14*). Myofibril samples were diluted to 3.2 mg/mL, and then 1 vol of each sample was immediately combined with 0.5 vol of a sample buffer/tracking dye solution (3 mM EDTA, 3% SDS, 30% glycerol, 0.003% pyronin Y, and 30 mM Tris-HCl at pH 8.0) and 0.1 vol of 2-mercaptoethanol. The samples were then heated at 50 °C for 20 min. Fish myofibril samples that were used to examine the heating conditions were supplemented with a protease inhibitor mixture containing 100 μg/mL of E-64, leupeptin (Peptide Institute, Osaka, Japan), and phenylmethanesulfonyl fluoride (Wako Pure Chemical Industry, Osaka, Japan) before being combined with the sample buffer/tracking dye solution. The ratio of myofibril:protease inhibitor mixture was 100:1 (v/v).

Preparation of SDS−PAGE Samples of Whole Muscle. Whole muscle samples were prepared by using a modification of the method of Bechtel and Parrish (*15*). A 0.1 g amount of muscle was knife-minced, and 2 mL of a solution (2% SDS and a 10 mM sodium phosphate buffer at pH 7.0) or 1.94 mL of the same solution and an inhibitor mixture containing 0.02 mL each of 100 μg/ml of E-64, leupeptin, and phenylmethanesulfonyl fluoride was added according to the method of Kubota et al. (*16*) to prevent proteolysis. The sample mixture was then homogenized with a motor-driven glass homogenizer (Iuchi Co., Osaka, Japan) at 2000 rpm/min and finally centrifuged at 1600*g* for 5 min at 25 °C to remove the trace of insoluble components. The resulting supematant was combined with the sample buffer/tracking dye solution and heated in the same way as that described for the myofibril samples. In accordance with the method of Kubota et al. (*16*), whole muscle samples were also heated twice at 100 °C for 2 min, once before and once after adding 2-mercaptoethanol.

Electrophoresis. To detect the protein bands smaller than the myosin heavy chain band, 12% polyacrylamide (acrylamide:bisacrylamide = 37:1), slab separating gel with 5% polyacrylamide (acrylamide:bisacrylamide = 100:1) stacking gel was used. A 5% gel (100:1), a 3−7.5% gradient gel (100:1), and a 3−5% gradient gel (100:1), all without stacking gels, were each used to identify the most suitable gel combination for separating of α- and β-connectin in fish muscle. Glycerol was added to the 7.5% and 5% acrylamide solutions to make a final concentration of 15% glycerol to facilitate the formation of a gradient. The other chemicals in the gel solution were the same as those used in the method of Huff-Lonergan et al. (*13*). The gradient gels were made with a model 385 gradient former (Bio-Rad Laboratories, Hercules, CA). Each gel (8 cm long × 7.3 cm tall × l.5 mm thick) was mn on a Bio-Rad Mini Protean II system at 3.5 mA/gel for 18 h with a running buffer of 25 mM Tris, 0.192 M glycine, 0.1% SDS, and 0.07% 2-mercaptoethanol. The amount of protein applied to the gels is given in the figure legend. The gels were stained overnight in 0.1% Coomassie brilliant blue R-250, 40% methanol, and 7% glacial acetic acid and destained in the same solution without Coomassie brilliant blue R-250. The intensity of the protein bands in the gels was compared by using Scion Image computer software.

RESULTS AND DISCUSSION

***25%* Gel SDS−PAGE Analysis.** The results of the SDS−PAGE analysis of the whole muscle and myofibril samples prepared from beef round meat are shown in **Figure 1**. It is clear that separation of the α- and β-connectin bands in beef round muscle was possible with 5% (100:1) SDS−PAGE. The 0-day whole muscle sample showed a quite faint band of β-connectin, while the majority was α-connectin. It was gradually degraded to β-connectin with increasing storage time. A 1200 kDa product (*17*), the degradation product of beef α-connectin, appeared in the 1-day sample. On day 7, the band of α-connectin disappeared, and that of β-connectin remained

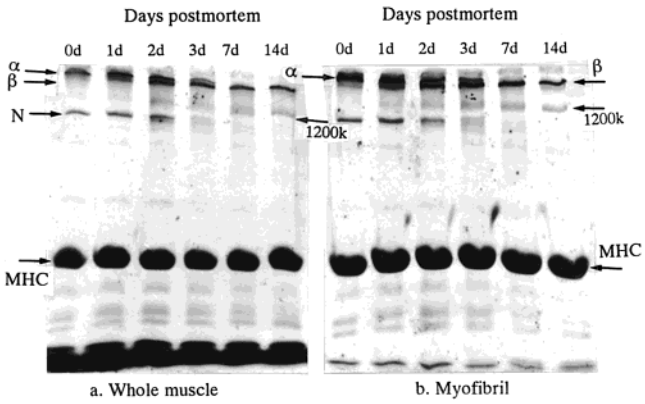

Figure 1. SDS–PAGE analysis on 5% polyacrylamide gel of the whole muscle and myofibril from beef. Samples were taken from beef round meat 0, 1, 2, 3, 7, and 14 days postmortem. A 60 μg amount of protein was applied to each lane. α, α-connectin; β, β-connectin; N, nebulin; 1200k, 1200 kDa product; MHC, myosin heavy chain.

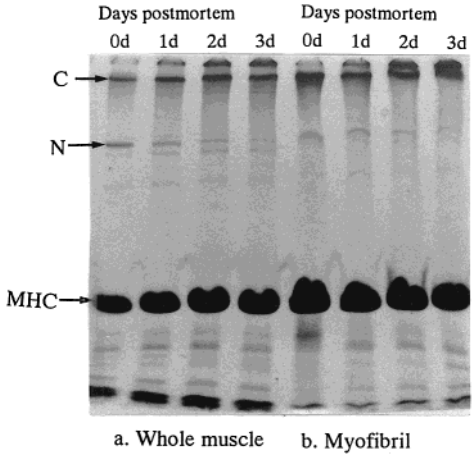

Figure 2. SDS–PAGE analysis on 5% polyacrylamide gel of the whole muscle and myofibril from jack mackerel *(T. japonicas).* Samples were taken from the white dorsal muscle 0, 1, 2, and 3 days postmortem. A 60 μg amount of protein was applied to each lane. C, connectin; N, nebulin; MHC, myosin heavy chain.

and did not decrease until day 14. On the other hand, the degradation of nebulin began on day 2, and its band became very faint after day 3. These two proteins in the myofibril samples showed patterns very similar to those of the whole muscle samples throughout the storage period. Thus, for beef round meat, both types of samples provided a valid means for evaluating the rate of degradation of the intact forms of these two proteins. This result concurs with that in the report of Huff-Lonergan et al. (*10*). The results of SDS−PAGE of the whole muscle and myofibril samples prepared from jack mackerel in 5% (100:1) gel were almost the same (**Figure 2**). Only one band for connectin appeared from day 0 to day 3, and the band for nebulin became faint after day 1. These results suggest that separation of the α- and β-connectin bands in fish muscle was not possible in 5% (100:1) gel by SDS−PAGE or that all of the intact α-connectin was degraded to β-connectin during the preparation of the SDS−PAGE samples. In addition, no difference was apparent in the SDS−PAGE patterns between the whole muscle and myofibril samples, so it was decided to use the whole muscle samples prepared with a protease inhibitor mixture to search for the best gel composition to separate the α- and β-connectin in fish muscle.

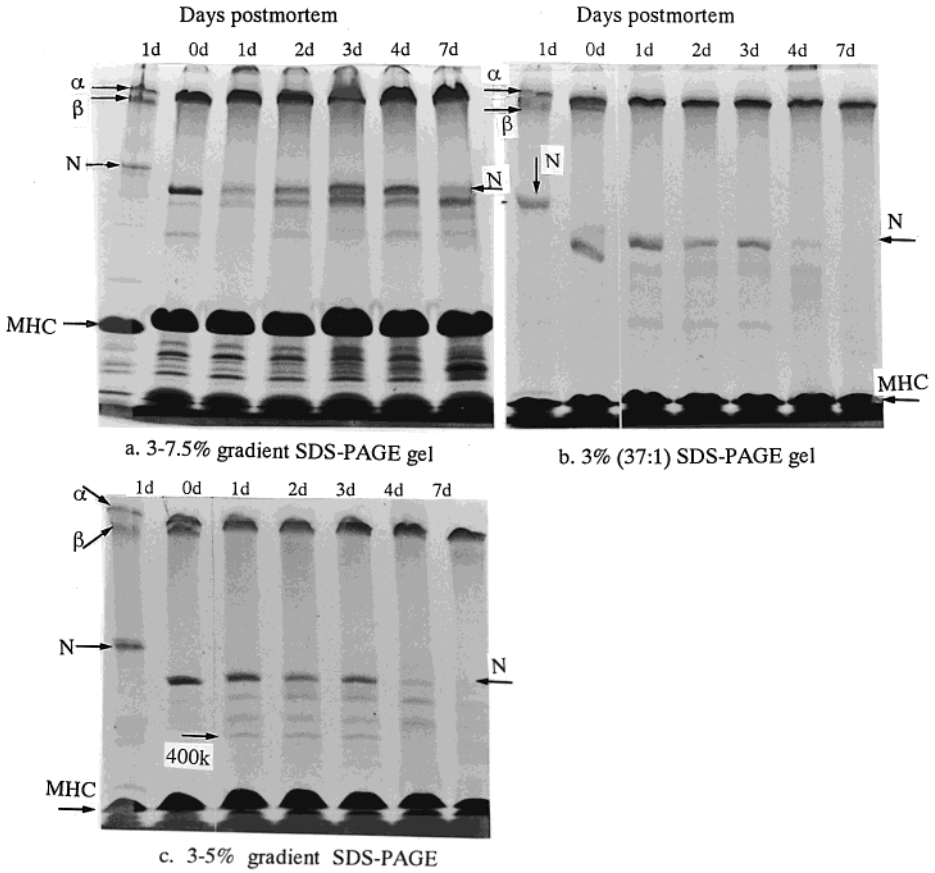

Figure 3. Comparison of the SDS−PAGE analysis with various polyacrylamide concentrations. Samples were taken from the white dorsal muscle of jack mackerel *(T. japonicas).* The first lane (1 d) in each gel shows the myofibril sample taken from beef round meat stored for 1 day. A 115 μg amount of protein was applied to each lane. α, α-connectin; β, β-connectin; N, nebulin; 400k, 400 kDa product; MHC, myosin heavy chain.

3−7.5% Gradient, 3%, and 3−5% Gradient Gel SDS−PAGE Analysis. In the 3−7.5% gradient gel (**Figure 3**a), the α- and β-connectin bands could be separated in the beef myofibril sample (first lane), but not in the fish whole muscle sample. Connectin from the day 0 fish muscle migrated as a very closely spaced doublet band in the 3% gel (**Figure 3b**, second lane), but there was not enough space to distinguish the α-connectin band from the β-connectin band. However, in the 3−5% gradient gel (**Figure 3c**), connectin was clearly divided into two bands in the 0-day sample. Additionally, the fish β-connectin band was in almost the same position as that of the beef in the 3−5% gel. It was conceivable that the mobility of beef and fish β-connectin was identical, considering the previous observations of Seki and Watanabe (*5*) and Hu et al. (*6*); they found that the mobility of β-connectin from carp or goldfish muscle corresponded to that from rabbit muscle or chicken breast muscle. Fish nebulin was faster in mobility than beef nebulin. Seki and Tsuchiya (*8*) have reported that the molecular masses of α- and β-connectin and nebulin from carp muscle are 2400, 2100, and 580 kDa, respectively, by SDS gel electrophoresis using cross-linked myosin heavy chains as standard according to the method of Maruyama et al. (*18*). They also reported that the 400 kDa product (*19*), supposed to be a degradation product of connectin, was detected in 1-day stored carp myofibrils at 15 °C. The band just above that of the myosin heavy chain (**Figure 3c**, third lane) appeared on day l and might correspond to the 400 kDa product in their report, as indicated by comparison with the positions in the gel. It might be the degradation product of fish α-connectin, which was confirmed by western blotting (data not shown). This new band disappeared after 5 days of storage, in good agreement with the result of Seki and Watanabe (*5*) for carp myofibrils. It was thus found that separation of the α- and β-connectin bands in fish could be achieved with a 3−5% gradient gel. To ensure reproducible separation, the gel was polymerized at 35 °C according to the method of Gressel et al. (*20*) for at least 5 h.

Heating Conditions for the SDS−PAGE Samples. The SDS−PAGE samples were heated at 50 °C for 20 min in this study. This temperature might not be enough to denature the proteases, but it was sufficient to activate them even if the protease inhibitors were contained. Kubota et al. (*16*) heated samples containing an inhibitor mixture at 2100 °C for 2 min, mixed in 2-mercaptoethanol in order to prevent thiol protease activation, and then heated the samples again at 100 °C for 2 min. The SDS−PAGE patterns of samples prepared under different heating conditions were compared to determine the most suitable heating temperature. As shown in **Figure 4** (lanes 3 and 4), heating at 100 °C for 2 min clearly caused a decrease in the connectin band, and heating the samples twice at 100 °C for 2 min, before and after addition of 2-mercaptoethanol, caused a greater decrease than heating once; the amount of the connectin, which was calculated from the peak area of the connectin bands, was about one-third (33.7 ± 4.4%) and one-fourth (24.8 ± 11.1%), respectively, of that found when the samples were heated at 50 °C for 20 min (**Figure 4**, lane 2). The nebulin band, however, showed the same intensity, regardless of the heating conditions. This result is consistent with the fact that connectin was unstable in the SDS solution at room temperature, while nebulin was completely stable under the same condition (*21*). Kubota et al. (*16*) used a solution 1% SDS containing 8 M urea to dissolve the muscle and heated at 100 °C for 2 min, so it is possible that some degradation of connectin

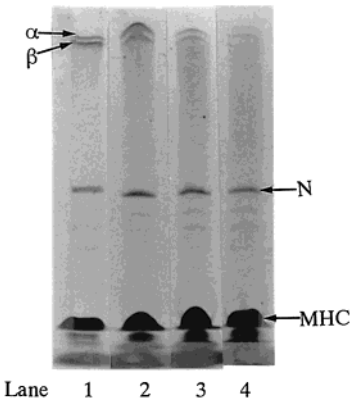

Figure 4. Effect of heating conditions on the SDS–PAGE samples. SDS–PAGE analysis was carried out with 3–5% polyacrylamide gel. Lane 1, myofibril sample (60 μg of protein) heated at 50 °C for 20 min; lane 2, whole muscle sample (115 μg of protein) heated at 50 °C for 20 min; lane 3, whole muscle sample (115 μg of protein) heated at 100 °C for 2 min; lane 4, whole muscle sample (115 μg of protein) heated twice at 100 °C for 2 min, before and after the addition of 2-mercaptoethanol. All of these SDS–PAGE samples contain 7% 2-mercaptoethanol. α, α-connectin; β, β-connectin; N, nebulin; MHC, myosin heavy chain.

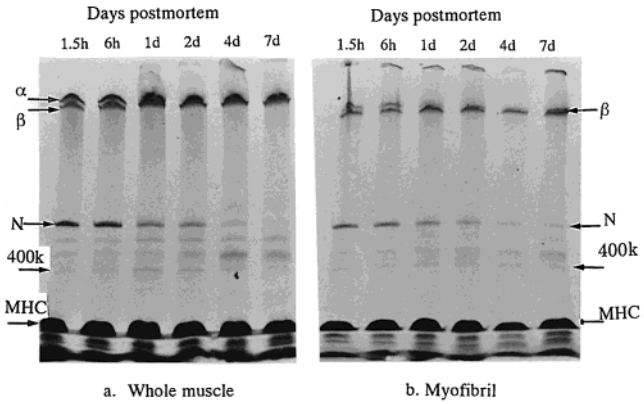

Figure 5. Changes in the SDS–PAGE patterns (3–5% gel) of the whole muscle and myofibril samples of jack mackerel *(T. japonicas)* stored at 4 °C. Samples were taken from white dorsal muscle 1.5 h, 6 h, 1 d, 2 d, 4 d, and 7 d postmortem. A 115 μg amount of protein was applied to each lane of whole muscle. A 60 μg amount of protein was applied to each lane of myofibril. α, α-connectin; β, β-connectin; N, nebulin; 400k, 400 kDa product; MHC, myosin heavy chain.

occurred. Connectin and nebulin in the 1.5 h postmortem myofibril sample (**Figure 4**, lane 1) were estimated to account for approximately 8.9% and 3.6%, respectively, of the total myofibrillar proteins, which were calculated as the ratio of each band's peak area to the total bands' peak area of myofibrillar protein. Their sum (12.5%) is in good agreement with the value of 13% of the total myofibrillar proteins from seven fish species reported by Seki and Watanabe (*5*), who isolated connectin and nebulin by gel filtration of the SDS extract of the myofibrils and estimated by the biuret method. On the other hand, connectin and nebulin in the whole muscle sample were estimated to account for approximately 10.1% and 4.1%, respectively, of the total muscle proteins, which were calculated in the same manner as in the myofibril sample. If the myofibrillar proteins comprised 70% of the total muscle proteins, the figures for connectin and nebulin should respectively be 14.4% and 5.9%, and their sum should be 20%. Further research is needed to clarify this discrepancy. Regardless of the sample type, the ratio of connectin to nebulin was almost the same,

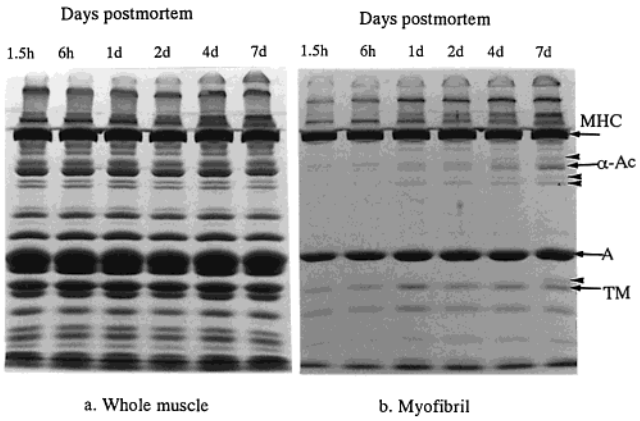

Figure 6. Changes in the SDS–PAGE patterns (12% gel) of the whole muscle and myofibril samples of jack mackerel *(T. japonicas)* stored at 4 °C. Samples were taken from white dorsal muscle 1.5 h, 6 h, l d, 2 d, 4 d, and 7 d postmortem. A 115 μg amount of protein was applied to each lane of whole muscle. A 60 μg amount of protein was applied to each lane of myofibril. The arrowheads show new protein bands produced during storage. MHC, myosin heavy chain; α-Ac, α-actinin; A, actin; TM, tropomyosin.

Table 1. Changes in the Amounts of Connectin[a] and Nebulin during Storage

	whole musle		myofibril	
storage time[b]	connectin (%)[b]	nebulin (%)	connectin (%)[b]	nebulin (%)[b]
1.5 h	100	100	100	100
6 h	97.2 ± 9.9	102.2 ± 6.5	94.5 ± 5.0	103.4 ± 8.9
l d	82.3 ± 20.5	71.8 ± 23.6	85.1 ± 1.9	58.4 ± 2.1
2 d	85.9 ± 1.2	41.2 ± 3.6	82.9 ± 12.0	38.5 ± 9.8
4 d	79.1 ± 13.6	28.6 ± 17.5	70.2 ± 18.8	19.1 ± 10.6
7 d	82.7 ± 15.7	14.5 ± 14.0	78.6 ± 7.7	12.4 ± 10.8

[a] Connectin was the sum of α- and β-connectin. [b] Each value was calculated as the ratio of the amount of connectin or nebulin in each sample to that in the 1.5 h postmortem sample and represented as means + SD (n = 3).

i.e., 2.47:1 in the myofibril sample and 2.46:1 in the whole muscle sample, respectively. The SDS−PAGE pattern of the myofibril sample prompts the suggestion that α-connectin was degraded during the preparation of myofibril since the β-connectin band exceeded the α-connectin band in staining intensity in comparison with the balance in the whole muscle sample.

Changes in the SDS−PAGE Patterns of Fish Muscle during Storage. The changes in SDS−PAGE patterns of the whole muscle samples prepared from jack mackerel *(T. japonicus)* stored at 4 °C are shown in **Figure 5a**. Until 6 h postmortem, both bands of α- and β-connectin were present. After 24 h, only the β-connectin band remained, and the amount of connectin had decreased to about 80% of that at 1.5 h, although it remained almost steady until day 7 (see **Table 1**). The nebulin band did not show any change until 6 h postmortem, but after 24 h, it continuously decreased until day 7, when the amount of nebulin was only about 15% of that at 1.5 h (see **Table 1**). The myofibril sample showed changes in the amounts of connectin and nebulin during storage similar to those observed in the whole muscle sample (see **Table 1**). It was suggested that α-connectin was degraded to β-connectin during the preparation of myofibrils from the comparison of connectin bands in the whole muscle sample with those in the myofibril sample (**Figure 5a**,**b**). In addition, the 400 kDa product, which is believed to have been another degradation product of α-connectin, could be detected more clearly in the SDS−PAGE

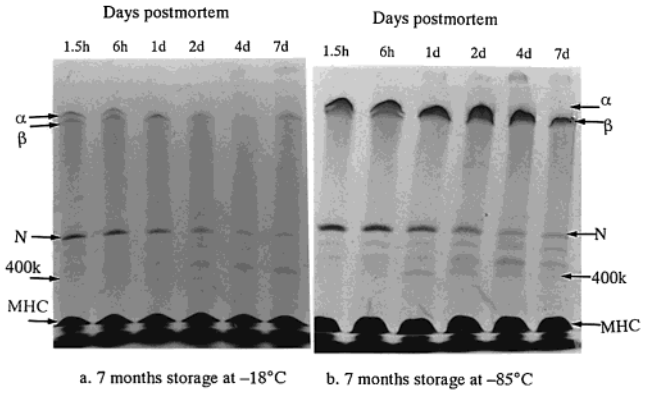

Figure 7. Effect of storage temperature on the stability of the whole muscle SDS–PAGE sample. The SDS–PAGE samples prepared in the same way as described in **Figures 5** and **6** were stored at –18 and –85 °C for 7 months. SDS–PAGE analysis was carried out with 3–5% polyacrylamide gel. A 115 μg amount of protein was applied to each lane. α, α-connectin; β, β-connectin; N, nebulin; 400k, 400 kDa product; MHC, myosin heavy chain.

pattern of the whole muscle sample than in that of the myofibril sample. Thus, the whole muscle sample was suitable to investigate the changes of connectin and nebulin in fish muscle during storage. With respect of the protein bands smaller than the myosin heavy chain band, several new bands appeared around the α-actinin band and increased the intensity during storage. In addition, another new band appeared on day 7 just above tropomyosin in the myofibril sample (**Figure 6b**). Further study is necessary to identify the origin of these bands.

Changes in the SDS−PAGE Patterns of Samples during Preservation. The patterns of the prepared SDS−PAGE samples that had been preserved at −18 °C for 17 months became unclear (**Figure 7a**), and the amount of connectin was only 4.8% of the whole muscle proteins in the 1.5 h postmortem sample. This is about half of the amount before preservation, although the amount of nebulin (4.2%) did not decrease. On the other hand, preservation at −85 °C (**Figure 7b**) did not have any effect on the amounts of connectin and nebulin, these being 10.9% and 4.4%, respectively, in the 1.5 h postmortem sample, which are almost the same as those just after preparation of the SDS−PAGE sample. It is obvious from these results that preserving the SDS−PAGE samples at −85 °C was better than that at −18 °C to obtain reproducible SDS−PAGE data.

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