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CHEMICAL CROSS-LINKING OF ACTIN AND MYOSIN SUBFRAGMENT-1 AND EFFECT OF PARATROPOMYOSIN ON THE INTERACTION OF ACTIN AND MYOSIN SUBFRAGMENT-1

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Backgrounds and Objectives

The interaction between myosin and actin in muscle cells influences not only the rigidity of postmortem muscle architecture but also the properties of heat-induced gels of these proteins in meat products[6]. However, there is a controversy about the intensity of the interaction between actin and myosin in postmortem muscle. Fujimaki et al.[2] have found that "myosin B" prepared from postmortem muscle is more easily dissociated into its components than that of muscle immediately after slaughter by the addition of 1-5mM ATP. Since then, many meat research workers have been fascinated with this interesting finding, so that it has been widely accepted that actin-myosin interaction is weakened during postmortem storage of muscle at cold temperatures. After the development of SDS-polyacrylamide gel electrophoresis, it was revealed that myosin B contains troponin, tropomyosin, α -actinin and other minor components in addition to actin and myosin[10]. Therefore, the necessity for investigating the effect of these minor components on the actin-myosin interaction in postmortem muscle has been required, in order to understand the nature of the interaction between constituent proteins of myofibril. Ikeuchi et al.[3] have found that the change of myosin-actin interaction during postmortem storage of muscle is not appreciably modified by the regulatory proteins, troponin, tropomyosin. Takahashi et al.[12] and Yamanoue and Takahashi[16] have indicated the implication of paratropomyosin in weakening the actin-myosin interaction in postmortem muscle.

As to the actin-myosin interaction itself, on the other hand, Ito et al.[4] have studied actin-activated heavy meromyosin ATPase activity prepared from muscle immediately after slaughter and from postmortem muscles, and have shown that the affinity of actin to myosin increases with increasing postmortem storage time at 0℃ by calculating Km values from double reciprocal plots (1 / ATPase activity vs. 1 / actin concentration) of actin activated - heavy meromyosin ATPase[1]. This result suggest that actin-myosin interaction itself increases for at least 1 week when muscles were stored at 0℃ and interaction may be weakened by other minor components of myofibrillar proteins.

Zero angstrom cross-linkers such as 1-ethyl 1-3-[3-(dimethylaminopropyl)-carbodiimide hydrochloride (EDC) has been successfully used for the formation of peptide bonds between two proteins which had contracted each other non-covalently[7]. Sutoh[9] have shown an actin-binding site of myosin molecule by using EDC, when actin and myosin forms rigor complex.

The objective of the present study was to investigate chemically the interaction of actin and myosin subfragment-1 (S-1) under various conditions by using zero angstrom cross-linker EDC. Especially, the difference in actin-myosin interaction between muscle immediately after slaughter and postmortem muscles was the major purpose of the present study. The effect of paratropomyosin on the interaction of actin and S-1 was also another major purpose of the present study.

Materials and Methods

Materials : Rabbits were anesthetized with pentobarbital prior to exsanguination. After dressing, rabbit carcasses were dipped in 10mM sodium azide for a few seconds, and then the carcasses were wrapped in polyethylene bags and stored in an ice box at 0℃ until use. Actin and myosin were prepared either from muscles immediately after slaughter (fresh muscle) or from muscles stored at 0 ℃ for 168 hr according to the procedure of Spudich and Watt[8] and Tonomura et al.[13] respectively. Preparation of S-1 was made by the method of Weeds and Taylor[15]. Paratropomyosin was purified from rabbit muscles by the method of Takahashi et al. [11].

Cross-linking of actin and S-1 : After dialysis against 100 vol of 0.1M NaCl, 10 mM imidazole-HCl (pH 7.0), 2 mM $MgCl_2$ in the presence or absence of 1-5 mM ATP at 0 ℃ overnight, 0.5 ml of S-1 (1mg / ml) and 0.5 ml of actin (1mg / ml) were mixed and the resulting mixture was incubated at 25 ℃ for 10 min in order to form rigor complex. Then, varying amounts of EDC dissolved in the above dialyzing buffer were added to the mixture (Concentrations of EDC in the reaction mixtures were 33, 16.7, 9.0, 5.0, 2.5, 1.0, 0.5 and 0.2 mM in order), followed by the incubation at 25 ℃ for 1hr. During the incubation, S-1 and actin molecules in rigor complex form intermolecular peptide linkage between amino and carboxyl groups of those proteins. The crosslinking reaction was quenched by the addition of approximately 10-fold excess of β -mercaptoethanol. To examine the effect of paratropomyosin on the chemical cross-linking of actin and S-1, purified paratropomyosin was added to the mixture of actin and S-1 (actin : paratropomyosin = 3 : 1 ; as weight ratio), and rigor complex formation and EDC reaction (33 mM) were conducted as described above.

Detection of chemically cross-linked rigor complex : Immediately after the cross-linking reaction was quenched, an equal volume of

2-fold concentration of sample buffer (1% SDS, 10 mM Tris-HCl, pH 6.8, 1mM β-mercaptoethanol, 1 mg/ml bromphenol blue, 20% glycerol) was mixed with the reaction mixtures. The resulting sample mixtures were electrophoresed on gradient gels (7.5-17.5 % polyacrylamide gels) containing 1% SDS according to the procedure of Laemmli[5]. Western blotting of SDS-PAGE of chemically cross-linked products in the presence or absence of paratropomyosin was performed as described by Towbin et al.[14]. The detection of main bands on PVDF membrane was made by using rabbit anti-skeletal myosin and anti-actin (Sigma), and western blot kit (PROTEIN DETECTOR ; KPL, Inc.).

Results and Discussion

To determine preferable condition for the formation of rigor complex, actin (42 kDa), S-1 and the reaction mixtures of S-1 and actin in 0.1 M NaCl at pH 7.0 after the treatment with varying concentrations of EDC were electrophoresed on gradient gels (7.5 -17.5 % polyacrylamide gels). S-1 preparation in this experiment showed two major bands (S1a and S1b, whose molecular weights were approximately 116 and 90 kDa respectively) and two minor bands, which supposedly be proteolytic fragments (S1f1 and S1f2) of S1a, in addition to the bands of myosin light chains. The intensity of actin and subcomponents of S-1 decreased, while four new bands appeared after the treatment with EDC. In addition, the intensity of the four bands increased with increasing the concentration of EDC. From the estimation of the molecular weight of these new bands (a, b, c and d, whose molecular weights were estimated to be approximately, 230, 160, 130 and 80 kDa), it is likely that band b is a complex of actin and S-1a (1:1 complex), whereas band c is derived from the binding of actin and S-1b (1:1 complex), although the bands b and c show double bands. However, it is obscure why the band b and c show double bands. The molecular weight of band d is smaller than that of S-1b, and the intensity of the band of myosin light chains, especially light chain-2, was decreased at any time when the band d appeared. These results indicate that the band d might be composed of one part of actin and two parts of myosin light chains. Band a having larger molecular weight than band b was clearly detected at 2.5 mM EDC, while the formation of bands b-d was detected even at 0.5 mM EDC. The difference in the sensitivity of protein complex b against EDC suggest that there is a delicate difference in the affinity of the protein complex between rigor complex and the complex produced by simple association of S-1 under the condition of low ionic strength. Therefore, band a might be an association product of the heavy chain moieties of S-1. Quite similar results were also observed in the case of rigor complex formed from actin (168 hr) and S-1 (168 hr). These results also inversely indicate that EDC treatment preferentially forms covalently bonded complex between actin and S-1 in rigor state.

These results indicate that chemical cross-linking reaction for analyzing the affinity of actin and S-1 in rigor complex may be less sensitive procedure than kinetic analysis of actin-activated heavy meromyosin ATPase[4]. However, the present result, at least, reconfirmed that the affinity of actin and myosin in postmortem muscle in not smaller than that of fresh muscle. In other words, the present results indicate that the weakening of actin-myosin interaction in postmortem muscle is not due to a simple weakening reaction at crossbridges of rigor complex. Therefore, there is a possibility that regulatory proteins other than tropomyosin-troponin system, such as paratropomyosin, may be involved in the modification of actin-myosin interaction during the development of the resolution of rigor. Therefore, to elucidate the effect of paratropomyosin on actin-myosin interaction, the extent of actin-myosin complex formation in the absence or presence of paratropomyosin by EDC reaction was investigated by using SDS-PAGE. As a result, the formation of new bands was reduced after the addition of paratropomyosin, while the intensity of S-1b was almost no change. Evidently paratropomyosin inhibited the formation of actin and S-1 complex by EDC reaction. The main bands on SDS-PAGE were identified by western blotting and the following immnostaining. This result indicates that paratropomyosin is involved in the weakening of actin-myosin interaction during the development of the resolution of rigor.

Contradiction between the result of Fujimaki et al. (1965) and that of Ito et al. (1978) regarding the actin-myosin interaction in postmortem muscle will be solved if myosin B contains paratropomyosin.

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