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Biomolecular Strategies for Preparation of High Quality *Surimi*-Based Products

– Review –

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Abstract

There exist two interesting phenomena in making seafood products from *surimi*. When salted *surimi* is kept at a constant low temperature (4~40°C), its rheological properties change from sol to gel, which is called "*setting*". Seafood processors can exploit changes that occur during *setting* in preparation of *surimi*-based products, because heating at high temperatures, after the pre-heating during the *setting* process, enhances the gel-strength of salted *surimi*. Contrarily, when salted *surimi* or low-temperature set gel is heated at moderate temperatures (50~70°C), a deterioration of gel is observed. The phenomenon is termed "*modori*". In the *modori* temperature range, heat-stable cysteine proteinases such as cathepsin B, H, L and L-like hydrolyze the myosins responsible for gel-formation, resulting in gel weakening *modori*. This article reviews molecular events occurring during gel *setting* that improve the quality of *surimi*-based products, and inhibition of *modori* by applying proteinase inhibitors. Application of recombinant protein technology to *surimi*-based products is introduced and its prospects for practical use are discussed.

Key words: *surimi*, heat-induced protein gel, *kamaboko*, *modori*, gel-softening, myosin, cystatin C, glycosylation

INTRODUCTION

Surimi, a water-leached and cryo-stabilized ground fish muscle paste, is a raw material for making gelled seafood products, such as *kamaboko* and imitation shellfish. There are two unusual thermal transformations that can occur during processing of *surimi*-based products. During low-temperature storage at 4~40°C for extended time periods (1 to 24 hours depending on temperature), salted *surimi* develops from a paste into a limber gel. The low-temperature gelation is called "*setting*". Cooking surimi at high temperatures, *e.g.* 85°C, after the *setting* process makes a stronger gel than cooking at the same temperature without the setting process. Thus, the *setting* process has been applied to the production of *surimi*-based products in seafood industry. On the other hand, *surimi* gel deteriorates when exposed to moderate temperatures of 50~70°C. The gel-weakening phenomenon, named "*modori*", is an unfavorable occurrence during producing *surimi*-based products. *Modori* is principally a result of proteolysis by endogeneous muscle proteinases (1). Utilizing food additives including proteinase inhibitors together and minimizing exposure time to *modori* temperatures are effective strategies for minimizing the gel-weakening. The above two interesting phenomena are closely related to myosin molecules in muscle cells (Fig. 1). This article reviews structural characteristics of fish myosin, *setting* of *surimi*, and prevention of disintegration of gel by proteinase inhibitors.

STRUCTURAL CHARACTERISTICS OF FISH MYOSIN

Myosin is a fibrous protein, which plays an important role in muscle contraction by interacting with actin which is another major muscle protein and has molecular mass of 42 kDa. Myosin accounts for no less than 60% of myofibril proteins in skeletal muscle, and myosin and actin account for approximately 80%. Myosin, as used in this article, refers to skeletal muscle myosin. Besides muscle contraction, myosin performs key roles in producing muscle food. Heated myosin develops gel elasticity by means of various molecular interactions. Myosin is a major protein in muscle responsible for the functional properties of meat and meat products (2).

Myosin is composed of a hexamer including two heavy polypeptide chains (MHCs) and two pairs of light polypeptide chains (MLCs) (Fig. 1b). Its gross structure com-

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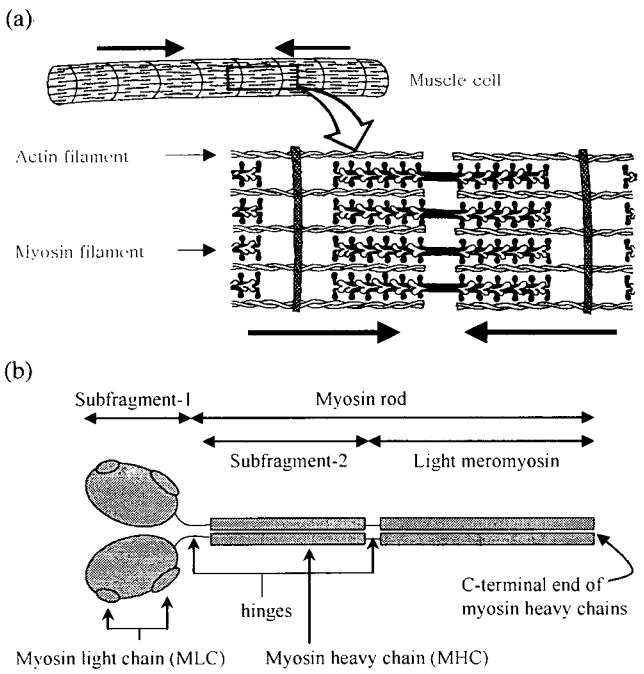

Fig. 1. Schematic diagrams of (a) myofibril and (b) myosin molecule.

prises two apparently identical pear-shaped heads and a long tail. The head is called subfragment-1, which is attached to the long tail called myosin rod. The myosin rod forms an alpha-helical coiled-coil. The molecular mass of myosin is approximately 520 kDa. Subfragment-1 has adenosine triphosphatase (ATPase) activity and actin-binding ability. Myosin rods have self-assembly ability to form the thick filament of skeletal muscle. Further details of the myosin molecule are described in details in biochemistry textbooks, such as Lehninger's Principles of Biochemistry.

In general, the physicochemical properties of fish myosin are similar to those of mammalian myosin. Fish myosin shows high homology with mammalian myosin in amino acid sequences (3-5). Nevertheless, its structural stability differs between fish and mammal. Fish myosin is inferior to mammalian myosin in thermal stability. Carp myosin starts denaturing at 30°C, but its rabbit counterpart at 40°C (6). Structural stability depends upon species and temperature of water in which the fish lives. Warm-water fish possess heat-stable myosin, whereas that of cold-water fish is unstable (7,8). Myosin from cold-water fish denatures even at low temperatures (4°C) during the purification process (7,9). A correlation of protein stability with habitat temperature of poikilotherm is likewise seen in the unfolding rate of myosin rod (10). Those trends are reasonable because proteins derived from the poikilotherm of fish are supposed to function efficiently, without denaturing, at swimming temperatures of fish.

Kakinuma et al. (11) found that controlling culture temperature of carp can alter the thermal stability of myosin upon spontaneous generation. Carp acclimated to cold-water (10°C) have myosin with lower stability than carp acclimated to warm-water (30°C). The report shows high environmental adaptability of fish. Kawabata et al. (5) suggested that the strength of coil formation near the C-terminal end determines the stability of myosin rod.

LOW-TEMPERATURE GELATION "*SETTING*" OF *SURIMI*

Salted *surimi*, fish meat paste, transforms to a gel even when maintained at a low temperature, around 40°C or at refrigeration temperatures. The term of gelation "*setting*" originated in Japan. It was originally named "*suwari*" in the Japanese language. Presently, the term, *setting*, seems to be more widely used. The "*setting*" process of fish meat paste differs with species (12). Myosin type is a major factor in the development of gel elasticity of muscle foods (2). *Setting* is mainly attributable to some molecular interactions among muscle proteins, especially myosin. Niwa et al. (13) reported that hydrophobic interactions are responsible for setting. Itoh et al. (14) stated that sulfhydryl-disulfide exchange reactions of actomyosin are also involved with *setting*. There is also a report describing how the interaction between myosin and actin, as assessed by the measurement of natural actomyosin (AM) Mg^{2+}-ATPase activity, is related to the *setting* (15). Formation of ε-(γ-Glu) Lys crosslinks between myosin molecules by endogenous transglutaminase (TGase) was reported by Seki et al. (16). Myosin molecules are polymerized by action of TGase (17). However, Nowsad et al. (18) showed that *setting* can occur even without the action of TGase. This demonstrates that the *setting* reaction includes multiple factors. Sano et al. (19) suggested that interaction among myosin rods induces gel elasticity. Visessanguan et al. (20) reported that myosin isolated from the cold-water fish, arrowtooth flounder, begins to unfold at 15°C and it shows an increase in surface hydrophobicity (that is, an increase in hydrophobicity) and a decrease in sulfhydryl content (that is, an increase in number of disulfide bonds) at temperatures above 20°C. Ogawa et al. (21) first proposed that the gel *setting* of *surimi* is initiated by unfolding of α-helices in actomyosin. The observation of actomyosin gel *setting* using Raman spectroscopy gave some interesting information (22). Slow unfolding of α-helical structures and exposure of hydrophobic amino acid residues occur during long-time

Table 1. Molecular events occurring during the *setting* reaction

Hydrophobic interaction	Niwa et al. 1981 (13)
Disulfide formation, gauche-gauche-trans form	Itoh et al. 1979 (14); Ogawa et al. 1999 (22)
ε -(γ -Glu) Lys formation by transglutaminase	Numakura et al. 1985 (17); Seki et al. 1990 (16)
Unfolding of α -helix	Sano et al. 1990 (19); Ogawa et al. 1995 and 1999 (21,22)
Actin-myosin interaction	Taguchi et al. 1978 (15)

incubation at the setting temperature of 40°C. The conformation of disulfide bonds inside set gels tends to be in the gauche-gauche-trans (*g-g-t*) form. Molecular events occurring during the *setting* reaction are summarized in Table 1.

In a previous section, the structure of fish myosin was shown to be fragile. The instability of fish myosin appears to be related to the *setting* response. One explanation is that unfolding of myosin molecules is an initial step in the formation of new molecular interactions (21). Thus, newly-formed molecular interactions such as hydrophobic interaction, S-S bond formation, and ε -(γ -Glu) Lys formation make myosins form a gel network. The higher the amount of unfolding of AM occurring during setting process, the stronger the gel (21).

MODERN *SURIMI* TECHNOLOGIES TO PREPARE HIGH QUALITY *SURIMI*-BASED PRODUCTS

Modern technologies for making high quality seafood products from *surimi* could allow active utilization of unutilized fish. Application of food-grade cryoprotectants successfully brought longer-term storage and better *kamaboko*-making properties to *surimi*.

Utilization of cryoprotectants

Several theories have been proposed for the mechanism of cryoprotection (23-25). Thermodynamic analysis demonstrated that sugars, polyols, amino acids, methylamines and lyotropic salts are preferentially excluded from contact with the surface of proteins in aqueous solutions, then the resulting interaction of solutes with proteins leads to the stabilization of protein molecules in non-frozen, aqueous systems (24). Gels may be degraded by freezing due to denaturation and aggregation of fish proteins that occur in the absence of cryoprotection (26). Actomyosin (AM) from ling cod (*Ophiodon elongatus*) was subjected to -10°C storage for 10 days in the absence or presence of cryoprotectants (sorbitol, sucrose, lactitol, and Litesse). Raman spectroscopic analysis revealed that the frozen AM had more α -helical structures with the addition of 4% cryoprotectants (27). These results suggest that the addition of cryoprotectants to AM may play an important role in stabilization of fish proteins against the deteriorative changes that are usually observed during frozen storage in the absence of cryoprotection (27).

Utilization of microbial TGase

As shown in Fig. 2, TGase participates in three catalytic reactions; (a) acyl-transfer reaction, (b) deamination, and (c) cross-linking reaction. Since it has been reported that ε -(γ -glutamyl) lysine cross-linking of proteins (Fig. 2c) enhances gel-strength in *surimi* (16,17, 28), use of microbial TGases has been proposed for enhancing gelation and viscoelastic properties of *surimi*-based products (29-35). ε -(γ -Glutamyl) lysine cross-links are assumed to be resistant to protease hydrolysis and could inhibit breakdown of myofibrillar proteins (36). The addition of microbial TGase has been shown to cause the cross-linking of myosin heavy chains and substantially increasing the gel strength of Mackerel *surimi* from 536.6 to 2,012.4 g×cm (34). Recently, a novel approach was developed for the high-level production of a microbial TGase from *Streptoverticillium* in *E. coli* (37). The direct expression of the TGase gene in *E. coli* cells did not cause overproduction, probably due to the harmful influence of TGase activity, therefore, a chemically synthesized TGase gene was designed and successfully overexpressed in *E. coli*. Since a large yield of TGase protein was obtained from the culture (200~300 mg/liter) (38), it will be possible to use the recombinant TGase after clearance of the food safety issue.

Utilization of food-grade gel-strengthening agents

Several food-grade agents were assessed for gel-strengthening of *surimi* from various fish species. Fibrinogen-thrombin combination, β-lactoglobulin rich specific whey

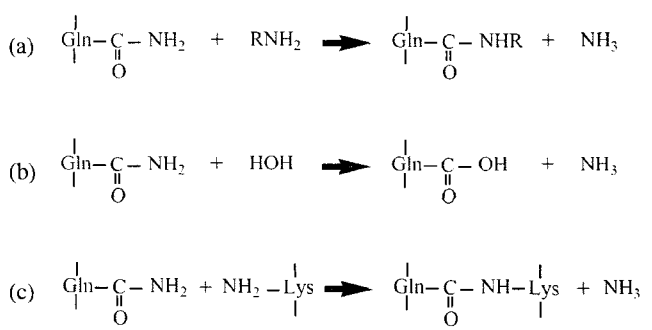

Fig. 2. General reactions catalyzed by transglutaminase. (a), acyl-transfer reaction; (b), deamidation; (c), cross-linking reaction.

protein, curdlan (Takeda Pharmaceutical Co. Ltd), polyphosphates and ascorbates were compared to the conventional egg-white-starch complex. Although some of them increased the gel strength and elasticity of *kamaboko* more than commercially required, none of them was more cost-effective than the conventional complex (39).

Recently, our understanding of the role of cellular proteinase involvement in protein degradation has reached the point that we can explain with more certainly the regulation and mechanism for controlling proteinase activities (40-49). Thus, food-grade-inhibitors have been successfully used to improve the gel strength of *surimi* by controlling proteolytic activity during *kamaboko* production. By adding bovine plasma proteins and egg-white, effective suppression of *surimi* autolysis was observed in Atlantic menhaden (41), Alaska pollock (41, 45), Pacific whiting (43,46,47,49) and arrowtooth flounder (42,45). Potato powder was effective in controlling autolysis of *surimi* from Pacific whiting (43,44,46,48), arrowtooth flounder (44) and trout (40). Indigenous and introduced legumes seed extracts also show promise for use as proteinase inhibitors during production of *surimi* from Pacific whiting (48). It is assumed that the effective suppression of autolysis by these compounds is due to a combination of proteinase inhibitory effects and their gelling properties (47,49). Application of food-grade inhibitors has the merit of being low cost, but large quantities of the agents are required to inhibit autolysis of *surimi* proteins. In some cases, the use of the agents is limited by the color (*e.g.*, red color of bovine plasma proteins). Therefore, more efficient and specific proteinase inhibitors need to be developed

PRODUCTION AND APPLICATION OF GENETICALLY PRODUCED CYSTATIN C, A CYSTEINE PROTEINASE INHIBITOR

Increased demand for *surimi*-based products has generated interest among researchers in the application of underutilized fish, such as roe-herring and late-run salmon. As a result, there is additional demand for better proteinase inhibitors for producing high quality *surimi*-based products from the underutilized fish, because most of them contain endogenous muscle proteinases, which weaken the gel strength of *surimi* gels by degrading myosin heavy chain (MHC) during cooking of *surimi* (50,51). Since major lysosomal proteinases in animal cells are papain-like enzymes (52,53), cystatin, an inhibitor of sulfhydryl proteinases, would be a useful ingredient to achieve this purpose. However, cystatin is hydrolyzed by endogeneous cathepsin D, an aspartyl proteinase; resulting in the loss of proteinase inhibitory activity of cystatin (54). Therefore, the improvement in conformational stability of this inhibitor is required to avoid the risk of degradation during the preparation process of *surimi*-based products from underutilized fish. Thus, it was proposed that the site-specific glycosylation of proteins occurred in yeast is a new approach to enhance their molecular stability against heating and proteolysis (55). Recently, a stable cystatin glycosylated with polymannosyl chains was successfully obtained by using two yeast expression systems (56,57). Chain-length of mannosylation of cystatin derived from *Pichia pastoris* was shorter and more homogeneous than that from *Saccharomyces cerevisiae*. The apparent molecular mass of the glycosylated cystatin from *P. pastoris* was 30 kDa on SDS-PAGE gel and the glycoprotein had a papain-inhibitory activity close to that of the non-glycosyl form (58,59). This review introduces some examples of the utilization of the genetically glycosylated cystatin.

Production of genetically glycosylated cystatin

Among four classes of proteinases: aspartic, serine, metallo, and cysteine proteinases, the one generally considered of greatest interest in *surimi* production has been the cysteine type including lysosomal proteinase cathepsin B, H, L, L-like, S and X. Consequently, it has become evident that cysteine protease inhibitors are effective in the prevention of the deterioration of protein-gel in *surimi*. Cystatin, a potent cysteine proteinase inhibitor, has offered a new insight into the process in which proteinase-inhibitor participates. Thus, a molecular based strategy was designed to prepare more stable bioactive inhibitor of the lysosomal cathepsins. Olden et al. (60) and Gu et al. (61) reported that carbohydrate moieties contributed to the protection of polypeptide chains of glycoproteins from proteolysis. Exploiting site-specific glycosylation of proteins that occur in yeast is a new approach to enhance their molecular stability against heating and proteolysis with aspartic, serine, and metallo proteinases. Thus, genetically glycosylated (polymannosyl) cystatins with and without polymannosyl chain were obtained and applied for the preparation of *kamoboko* and heat-induced gel (56). Fig. 3 shows a schematic model of a site-specific glycosylated polymannosyl cystatin. The yield of the protein was around 100 mg/L culture. The relatively high yield will reduce production costs and maintain the price of the final product within a consumer-acceptable range.

Enhanced gel strength of heat-induced roe-herring *kamaboko*

The polymannosyl cystatin was found to be appreciably more thermostable than the unglycosylated control. Heating the polymannosylated protein at 95°C for 30

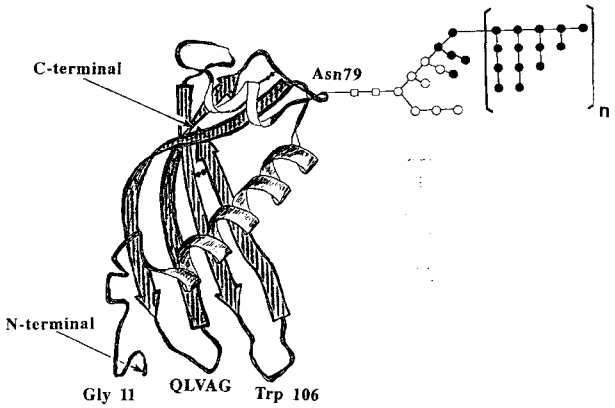

Fig. 3. Possible structural model for polymannosyl cystatin based on the crystal structure of egg-white cystatin. The polymannosyl chain was attached at the positions Asn_{79}. Gly_{11}, Trp_{106} and OLVAG (Gln_{69}-Leu_{70}-Val_{71}-Ala_{72}-Gly_{73}), which are assumed to be active sites against cysteine proteinases. □, ○ and ● indicate N-acetylglucosamine, mannose, and mannose attached in the *Golgi* apparatus, respectively.

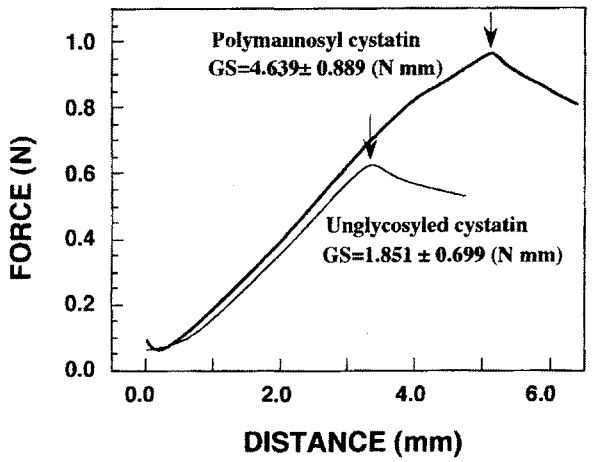

Fig. 4. Effect of addition of polymannosyl cystatin on gel breaking force of the herring *kamaboko*. Vertical arrows indicate breaking point of the tested *surimi* gels. GS represents gel strength of the tested gels.

min resulted in less than 40% reduction in the papain-inhibiting activity (57). Although it is reported that cystatin C is resistant to heating due to the tightly packed conformation (62), further enhancement of the thermostability was observed in the polymannosyl cystatin. The papain-inhibiting activity of the polymannosyl cystatin was better maintained than that of the unglycosylated control against attack from other types of proteinase. The papain-inhibiting activity of the polymannosyl cystatin retained about 50% of the initial activity after incubation with cathepsin D for 40 min, while the unglycosylated cystatin was rapidly inactivated with increasing incubation time (57). The polysaccharide chain could disturb the physical accessibility of cathepsin D to the proteolytic cleavage sites in the polymannosyl cystatin. Thus, a novel cysteine proteinase inhibitor having improved molecular stability has been developed (56,57).

The effect of the polymannosyl cystatin was assessed through application of it to roe-herring *surimi*. Fig. 4 shows the gel strength of the protein gel containing 10 g of the polymanosyl cystatin per gram protein. Without inhibitor, the herring *surimi* did not form a gel strong enough to be measured by the punch test. The texture of this gel was soft, gritty and somewhat mushy due to denaturation of the protein. Since a large amount of proteinases remained in the herring *surimi*, the gel formation was ruined by proteolysis of MHC. The addition of the polymannosyl cystatins to *surimi* was effective in increasing the punch force values. The herring *kamaboko* gel with the polymannosyl cystatin revealed 2.5 times higher gel strength than that with the unglycosylated cystatin. Thus, it was demonstrated that the polymannosyl cystatin effectively inhibited the degradation of MHC in roe-herring *surimi* during cooking (57). Recently, a Taiwan group reported a very similar results using polymannosylated chicken cystatin in yeast (63), and the polymannosyled cystatin markedly improved the gel strength of mackerel *kamaboko* (64). As a conclusion, the application of molecular biological technique for modifying a naturally occurring inhibitor, making a polymannosyl cystatin, facilitates the production of *surimi* from underutilized fish which should be expected to have a beneficial economic impact on the seafood industry.

CONCLUSION

Surimi proteins undergo some transformations during the *setting* process. Structural changes of myosin are mainly involved in some of the transformations occurring during *setting*. A slow unfolding of α-helix structures occurs in long-time incubation of *setting*. Hydrophobic amino acid residues are exposed to solvent on the surface; thus resulting in formation of hydrophobic interactions among molecules. In the set gel, some disulfide bonds are formed and their major conformation is *g-g-t* form. Action of endogenous or exogenous TGase accelerates the formation of set gel and enhances the gel strength of *surimi*-based products. Another concern is the deterioration of the protein gel network at *modori* temperatures. Many studies have been performed to prevent cooked gels from deteriorating. A prospective agent to prevent gel deterioration could be cystatin C, a potent cysteine proteinase inhibitor. Polymannosyl cystatin was effective in improving gelling properties of roe-herring *surimi*. The effect was achieved by strong inhibition of endogeneous sulfhydryl proteinases by polymannnosyl

cystatin with enhanced molecular stability. *Kamaboko* gel with a good texture can be prepared from roe-herring by adding polymannosyl cystatin. The polymannosyl cystatin could also be applicable to other underutilized fish such as late-run salmon and arrowtooth flownder.

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