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Glycosylation Modification Improved the Characteristics of Recombinant Chicken Cystatin and Its Application on Mackerel Surimi

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The recombinant and glycosylation chicken cystatins were expressed and secreted in the broth of *Pichia pastoris* X-33 transformant with apparent molecular masses (*M*) of 14 and 55 kDa, respectively. The glycosylation cystatin (glycocystatin) contained a polysaccharide chain that was composed of 50 DP of mannose residues. Because of the polymannosyl chain, the inhibitory ability in glycocystatin was 90.8% of recombinant cystatin. In addition to freeze−thawing stability, the thermal and pH stabilities as well as the susceptibility of glycocystatin were also enhanced. Both cystatins could improve the mackerel surimi gel by inhibiting the gel softening, which was derived from the hydrolysis of catheptic cysteine proteinases. Despite the additional amount of glycocystatin (8 units), twice that of recombinant cystatin, the 40 and 15% increases in breaking force and deformation of gels were also observed. Accordingly, the surimi gel was further improved by enhancing the stability of chicken cystatin.

KEYWORDS: ***Pichia pastoris* X-33; chicken cystatin; glycosylation modification; characterization; surimi**

INTRODUCTION

Fish mince, also termed surimi, is solely made from fish skeletal muscle. After the mince is chopped, detendoned, and leached, the gel-forming ability of fish mince could be much improved. During salt grinding of fish mince, some unique characteristics such as viscosity, elasticity, water-holding and gel-forming capacities were developed. The unfolding of an actomyosin (AM) molecule in the presence of salt leads to exposure of more functional groups from the inside helical structure of myofibrils. Hence, a firm network structure could be formed by the rearrangement or cross-linking of unfolded AM prior to boiling. These functionalities make it a valuable ingredient or base component for a broad range of reconstituted food products (*1*, *2*). However, the severe hydrolysis of endogenous proteinases toward myofibrillar proteins will cause detrimental effects on the gel-forming ability of some surimi such as mackerel (*3*, *4*), Pacific whiting (*5*), and chum salmon (*6*). Because of the disintegration of major cross-linkages, surimi always failed to form a satisfactory gel. Although the leaching process has long been employed in surimi manufacturing, it could not completely remove the proteinases or organelles, which are tightly bound to the myofibrils (*4*). In mackerel, the catheptic cysteine proteinases that exist in lysosomes are considered to be the major factor affecting the gel texture (*1*−*4*). Jiang et al. (*3*) and Ho et al. (*4*) reported that the high activities of lysosomal cathepsins B and L were still left in surimi even after freezing, salt grinding, and/or setting treatments. The remaining proteinases could severely hydrolyze myosin and consequently soften the gel strength. Hence, the inhibition of catheptic proteinases by using E-64 (1-*trans*-epoxysuccinyl-leucylamino-4-guanidinobutane) significantly reduced the proteolysis of the myosin heavy chain (MHC) and improved the gel quality of the surimi. A member of the cystatin superfamily, L-kininogen, obtained from porcine plasma, also exhibited a preventive effect on the gel softening (*7*).

The cystatin superfamily can be subdivided into four groups. They are stefins, cystatins, kininogens, and phytocystatins. In general, they are small proteins with molecular masses of about 10 kDa, except for kininogen (60−120 kDa), and widely distributed in various organisms. From the natures of specific cysteine proteinase inhibitors (*8*, *9*), the cystatins seem to have a high potential in the application of food processing and protection of agricultural crops. However, the low yields and complicated procedures in the purification of cystatins from plants or animal tissues increase the running cost and make the purification time-consuming (*10*). Thus, large-scale production by biotechniques is in high demand. In our previous studies, two cDNAs encoding chicken lung cystatin with or without N-glycosylation (N-Q-^{108}I→N-Q-^{108}T) have been successfully cloned and expressed in *Pichia pastoris* X-33 transformants in the soluble form (*11*, *12*). The methylotrophic yeast harboring a glyceraldehyde-3-phosphate dehydrogenase promoter is recognized as a consecutive and high-level expression host of recombinant proteins (*13*−*15*). Furthermore, this yeast strain also provides an ideal host for expressing the eukaryotic proteins as well as undergoing posttranslation modification.

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Although the recombinant chicken cystatin produced by *Escherichia coli* had characteristics quite similar to those of the wild type and could effectively inhibit the gel softening of mackerel surimi (*16*, *17*), the safety for human consumption still needs further investigation. Yeast is therefore considered to be an alternative host for high-level expression of recombinant proteins instead of *E. coli*. Jiang et al. (*12*) had indicated that the glycosylation modification of recombinant cystatin within the *P. pastoris* expression significantly improved the freezing stability upon six cycles of freeze−thawing. This study aims to investigate the effects of glycosylation on the other characteristics of chicken cystatin (glycocystatin) expressed in *P. pastoris* X-33 transformant and to evaluate its application on the mackerel surimi.

MATERIALS AND METHODS

Materials. Papain (2-fold crystallized), protease (also termed as pronase E, from *Streptomyces griseus*; EC 3.4.24.31), and E-64 were purchased from Sigma Chemical Co. (MO). Benzyloxycarbonyl-phenylalanylarginine-7-(4-methyl)coumarylamide (Z-Phe-Arg-MCA) and N-glycosidase F were the products of the Peptide Institute (Osaka, Japan) and Boehringer Mannheim GmbH (Mannheim, Germany), respectively. Live mackerel (*Scomber australasicus*) were obtained from a local fish market in Keelung, northern Taiwan.

Isolation and Cultivation of *P. pastoris* X-33 Transformants. Recombinant *P. pastoris* X-33 carrying the chicken cystatin and mutant cystatin (N-Q-^{108}I→N-Q-^{108}T) for N-glycosylation in-frame cDNAs was constructed as described by Chen et al. (*11*) and Jiang et al. (*12*). These two recombinants were streaked on zeocin-YPDS agar plates (20 g of tryptone, 10 g of yeast extract, 20 g of dextrose, 1 mol of sorbitol, 20 g of agar, and 100 mg of zeocin in 1 L of distilled water). After cultivation at 30 °C for 2−3 days, single colonies were selected and inoculated in 20 mL of zeocin-YPDS broth (20 g of tryptone, 10 g of yeast extract, 20 g of dextrose, 1 mol of sorbitol, and 100 mg of zeocin in 1 L of distilled water, pH 7.0). After the colonies were activated in a 30 °C shaking incubator overnight (250 rpm), 10 mL of each culture was inoculated in 500 mL of YPD broth (20 g of tryptone, 10 g of yeast extract, and 20 g of dextrose in 1 L of distilled water, pH 7.0) for 2−3 days. The crude recombinant and glycosylation chicken cystatins were obtained by 10 min of centrifugation at 4500*g* and subjected to further purification according to the methods of Chen et al. (*11*) and Jiang et al. (*12*).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS−PAGE). Cystatins in dissociating buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 3% SDS, 0.002% bromophenol blue, and 5% β-mercaptoethanol) were heated at 95 °C for 5 min. SDS−PAGE was performed on a 15% polyacrylamide slab gel according to the method of Laemmli (*18*). After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250 (*19*) and periodic acid-Fuchsin (*20*) solutions for protein and saccharide, respectively. Low molecular mass (*M*) calibration kit (phosphorylase b, 94 kDa; albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.4 kDa) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and protein ladders with a *M* of 10 kDa (Life Technologies, Inc., Gaithersburg, MD) were used as protein markers.

Protein Concentration and Saccharide Content. The protein concentration was determined by the dye-binding method (*21*). Bovine serum albumin was used as a standard protein. The total saccharide content of the glycocystatin was measured with the phenol−sulfuric acid reaction (*22*). The mannose was employed as a standard saccharide.

Assay of Inhibitory Activity. Papain was used as proteinase for the assay of inhibitory activity of cystatins, which was further purified according to the method of Machleidt et al. (*23*). The concentration of papain was determined by active site titration with E-64 as described by Barrett and Kirschke (*24*). The inhibitory activity of cystatin was assayed indirectly by measuring the residual papain activity using fluorescence substrate Z-Phe-Arg-MCA. Papain in 0.2 M sodium phosphate buffer (pH 6.0), containing 4 mM cysteine and 2 mM ethylenediaminetetraacetic acid (EDTA), with or without cystatin, was preincubated at 40 °C for 10 min. The enzyme mixture (0.75 mL) was comprised of 5 μL of papain, 0.25 mL of 0.4 M sodium phosphate buffer (pH 6.0) containing 8 mM cysteine and 4 mM EDTA, 0.295 mL of distilled water, and 0.20 mL of cystatin. The reaction was started by adding 0.25 mL of 40 μM Z-Phe-Arg-MCA solution and was stopped by adding 1.0 mL of sodium acetate buffer containing 0.1 M sodium monochloroacetate (pH 4.3). The amount of liberated aminomethylcoumarin was determined by a spectrofluorometer at an excitation of 350 nm and an emission of 460 nm. One unit of inhibitory activity was defined as the amount of cystatin that could inhibit one unit of the proteolytic activity of papain, whereas 1 unit of proteolytic activity was defined as the amount of papain that could hydrolyze Z-Phe-Arg-MCA and release 1 μmol of aminomethylcoumarin within 1 min of reaction at 40 °C.

Assessment of Stability. The recombinant or glycosylation cystatins in 50 mM Tris-HCl buffer (pH 7.5) were incubated at 30, 40, 50, 60, 70, 80, 90, and 100 °C for 30 min. After they were cooled in ice water for 30 min, the remaining inhibitory activity was measured. For the pH stability, cystatins in various buffers (50 mM citric acid−Na_2HPO_4, pH 2.6−7.5; 50 mM Tris-HCl, pH 7.5−8.5; 50 mM glycine−NaOH, pH 8.5−10.5) were incubated at 25 °C for 30 min, and the residual activity of treated cystatins was then assayed.

The susceptibility to proteinase was investigated under the hydrolysis by pronase E (EC 3.4.24.31). Recombinant and glycosylation cystatins in 50 mM potassium phosphate buffer (pH 7.5) mixed with 20 μg of pronase E were incubated in a 30 °C water bath for 30 min. The inhibitory activity of cystatins was determined every 10 min during a 30 min digestion.

Effect of Recombinant and Glycosylation Cystatins on Mackerel Surimi Gel. Mackerel surimi was prepared using live mackerel dorsal muscle. After the mackerel was deboned, the mince was first washed with 4 volumes of chilled 0.4% $NaHCO_3$ solution, then with chilled water, and finally with 0.3% NaCl for 5 min in each washing cycle. The resulting mince was dewatered to a moisture content of 78% by 15 min of centrifugation at 1500*g* and then mixed with 4% sucrose, 4% sorbitol, and 0.2% polyphosphate (a mixture of 50% sodium tripolyphosphate and 50% potassium pyrophosphate). The NaCl-free surimi was packaged in polyethylene bags (2.0 kg/bag) and stored at −40 °C until use. For evaluating the effect of recombinant and glycosylation cystatins on the gel properties of mackerel surimi, frozen mackerel surimi was thawed to −3 °C in a refrigerator and mixed with 1, 2, 4, 8, and 12 units of cystatins. After the surimi was ground with 2.5% NaCl for 30 min, 3% potato starch was uniformly mixed with the ground surimi and then stuffed into a polyvinylethylene chloride tube (3.0 cm). All surimi gels were set in a 50 °C water bath for 90 min to investigate the effect of cystatins on the prevention of gel softening. After the gels were heated at 90 °C for 30 min, the resulting gels were cooled in ice water for 30 min and stored overnight in a refrigerator. The gel properties were then examined by punch test using a rheometer with a 5 mm ball type plunger. Ten determinations in breaking force (g) and deformation (cm) were collected for each treatment. The change in protein patterns of surimi gels was observed using a 10% SDS−PAGE as described above.

RESULTS AND DISCUSSION

Expression of Recombinant and Glycosylation Chicken Cystatins. A recombinant (*11*) and a N-glycosylated mutant (N-Q-^{108}I→N-Q-^{108}T) (*12*) cystatin genes were constructed in pGAPZαC vectors, respectively, with secretive α-factor pre-prosequence, and transformed into methylotrophic yeast, *P. pastoris* X-33. These two forms of chicken cystatins were secreted and expressed in the broth. They were purified and characterized.

According to SDS−PAGE, the *M* values of recombinant and glycosylation cystatins were estimated to be 14 and 55 kDa, respectively. The staining for saccharide revealed a positive band on glycocystatin (**Figure 1**, lane E), which corresponded to the protein (**Figure 1**, lane C) and activity-staining band (**Figure 1**, lane D). This result indicated that the polysaccharide chain

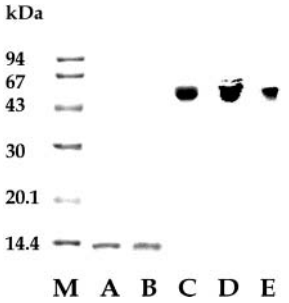

Figure 1. SDS–PAGE pattern of recombinant and glycosylation chicken cystatins expressed from *P. pastoris* X-33. Lane M, low molecular mass protein marker [phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa)]; lane A, recombinant chicken cystatin; lane B, N-glycosidase F digestion of glycocystatin; lane C, glycocystatin; lane D, activity (unhydrolyzed casein) staining of glycocystatin; and lane E, periodic acid-Fuchsin (saccharide) staining of glycocystatin.

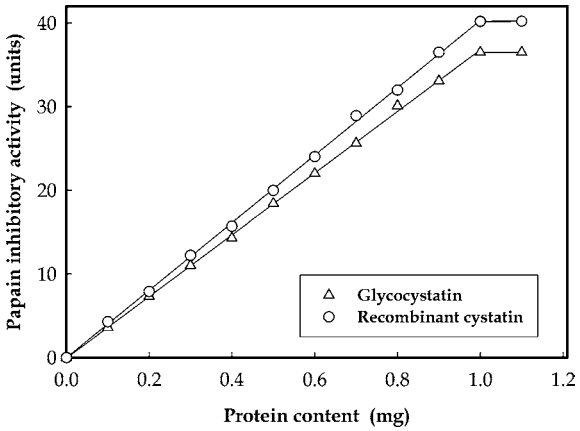

Figure 2. Comparison of the inhibitory abilities of recombinant and glycosylation chicken cystatins against papain. One unit of inhibitory activity was defined as the amount of cystatin that could inhibit one unit of the proteolytic activity of papain, whereas one unit of proteolytic activity was defined as the amount of papain that could hydrolyze Z-Phe-Arg-MCA and release 1 μmol of aminomethylcoumarin within 1 min of reaction at 40 °C.

had been covalently integrated into the potential N-glycosylation signal sequence, N-Q-^{108}T, in the carbonyl terminus of mutant chicken cystatin (**Figure 1**, lane B). The recombinant chicken cystatin expressed in yeast was unmodified. In our previous study (*12*), a polymannosyl chain with 40 degrees of polymerization (DP) was simply deduced from the gel filtration chromatography of glycocystatin. In this study, we reexamined the size of the polysaccharide by phenyl-sulfuric acid reaction and quantified it. It was found that 1 mol of glycocystatin contained a polyglycosyl chain with 50 mannose residues (data not shown). These data suggested that the glycocystatin expressed from *Pichia* is a 50 DP mannose-linking cystatin.

Comparing the specific inhibition activity of glycocystatin (36.5 units/mg protein) with that of recombinant cystatin (40.2 units/mg protein), it was about 90.8% inhibitory activity of the recombinant cystatin (**Figure 2**). Bode et al. (*25*) indicated that the chicken cystatin has a tightly bound structure and exposes three wedges toward the papain active cleft with a docking inhibition. Therefore, the N-polyglycosyl modification site was located neither within the interior of cystatin nor along the contact edges for inhibiting papain. In addition, this result also reflects the folding of either recombinant or glycosylation cystatins in *Pichia* host, as compared to that of wild-type.

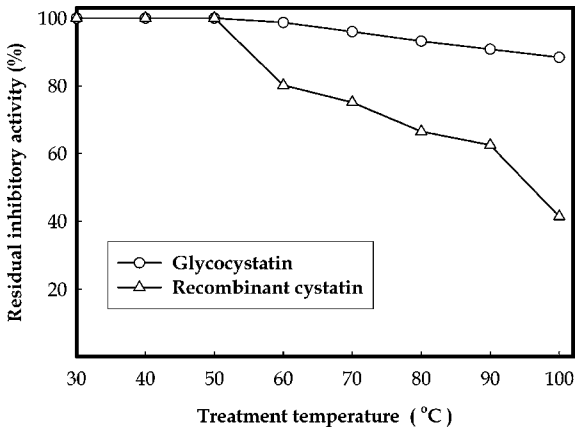

Figure 3. Comparison of the thermal stability of recombinant and glycosylation chicken cystatins incubated at 30–100 °C for 30 min.

Therefore, the inhibition ability was almost maintained in glycocystatin. Because the moderate glycosylation modification of proteins resembled the higher eukaryotes, the *Pichia* had been recently developed as a common yeast host for foreign protein expression. Unlike *Saccharomyces cerevisiae*, for example, expression of a hyperglycosylation mouse cystatin C contained 310 DP of mannose; this kind of modification consequently impairs most of the inhibitory activity of cystatin (*26*). However, the moderate polymannosyl modification of chicken (50 DP) or mouse (90 DP) cystatins expressed by *Pichias* always exhibited nearly full activity as their unmodified forms. The data suggested that the overextension of saccharide residues could hinder the function of protein and the expression of *Pichias* was more suitable for obtaining a soluble, active chicken cystatin.

Stability. As indicated in **Figure 3**, residual inhibitory activity of glycocystatin was significantly greater than that of recombinant after 30 min of incubation at 60−100 °C. About 90% residual inhibitory activity of glycocystatin was left, whereas only 40% activity remained in recombinant after 30 min of heating at 100 °C. Inserting a polyglycosyl chain into chicken cystatin increased the heat resistance 20−50% within 30 min of incubation at 60−100 °C.

The pH stability of both cystatins was also assessed in various pH buffers from 2.6 to 10.5. Within the tested pH, the residual inhibitory activities of both recombinant and glycosylated cystatins were almost not affected (data not shown). According to the data obtained, the chicken cystatin itself was very stable at a broad pH range. The effect of glycosylation on the pH stability of cystatin, therefore, could not be easily evaluated. For this reason, 0.1 N NaOH and HCl solutions were used to differentiate the resistance between these forms of cystatins, respectively. After 30 min of incubation at 25 °C, only 26.8 and 17.3% original activity was retained in NaOH and HCl treatment recombinant cystatin separately; however, those in glycocystatin were 46.4 and 44.7%. Because the attachment polymannosyl moiety could not directly provide buffering capacity against pH shift, it seemed likely that the attachment mannose residues affect the hydration state of cystatin.

The pronase E (EC 3.4.24.31) was a neutral serine protease obtained from bacterium and had broad digestibility toward various proteins. Hence, the susceptibility of cystatins expressed from yeast host was evaluated using this protease. According to **Figure 4**, more than 70% loss of inhibitory activity in recombinant cystatin was observed during the first 10 min of digestion while that of glycocystatin was only 30%. The efficient proteolysis of the cystatin protein part almost destroyed its

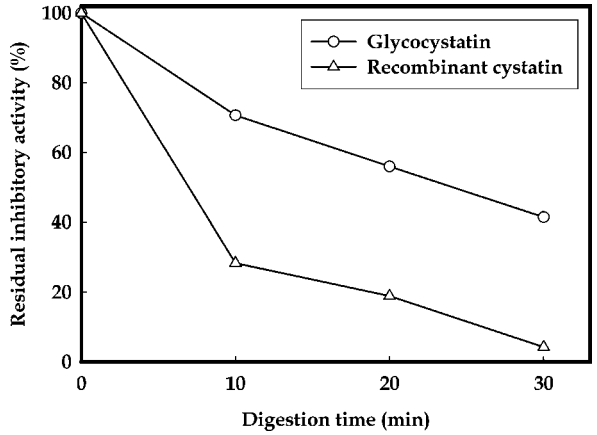

Figure 4. Susceptibility of recombinant and glycosylation chicken cystatins to the hydrolysis of pronase. Both cystatins in 50 mM potassium phosphate buffer (pH 7.5) mixed with 20 μg of pronase E were incubated in a 30 °C water bath for 30 min. The residual activity of cystatins in respective digestion solutions was determined as described in the Materials and Methods.

function after 30 min of incubation at 37 °C. In contrast, there was about half activity left in that composing a polysaccharide chain (**Figure 4**). The data obtained in the study revealed the remarkable stability of glycocystatin upon heating, a drastic pH shift, and proteolysis. Comparing with unmodified cystatin could find that the covalently linked polysaccharide chain significantly improved these properties. Jiang et al. (*12*) had reported that the effect of freeze−thawing on the activity of chicken cystatin was reduced by introducing a polymannosyl chain during yeast expression. The present study showed that the improvement of cystatin also includes thermal and pH stabilities and susceptibility in addition to multicycle freeze−thawing resistance.

Many reports had indicated that the glycosylation of proteins in yeast could increase thermal stability and present a positive relationship with the length of the attached carbohydrate (*26*, *27*). From the study of lysozyme, Kato et al. (*28*) found that the polymannosyl chain lowered the denaturation temperature of the active enzyme upon heating. Nevertheless, the refolding rate of heated glycosylation lysozyme to a proper structure was faster than that of the wild type, and consequently, it suppressed the self-aggregative denaturation of the unfolding molecule. The rapid and preferring correct conformational renaturation could explain the increase of apparently thermal stability of glycoproteins including chicken cystatin. Likewise, the mannose residues could also give a hindrance to other species proteases or play the role of a cushion to prevent the self-collision of core proteins during freezing, heating, and/or ion-induced dissociation (*12*).

Application of Recombinant and Glycosylation Cystatins on Mackerel Surimi. Crude recombinant and glycosylation chicken cystatins obtained from the culture broth of recombinant *P. pastoris* X-33 were dialyzed against 20 mM phosphate buffer (pH 7.0). To investigate their effect on the gel softening, cystatin samples were concentrated and added into mackerel surimi at a negligible volume. The NaCl-ground surimi with different additions (0−12 units) was incubated at 50 °C for 90 min to induce the hydrolysis of endogenous proteinases. After the punch test, the breaking force and deformation in control (cystatin-free) gels were shown to be 277 g and 6.0 mm, respectively, while those in gels supplemented with either recombinant or glycosylation cystatins were gradually strengthened. As shown in **Table 1**, when surimi was added with 4 and 8 units of recombinant and glycosylation cystatins, the breaking force and

Table 1. Breaking Force (g) and Deformation (mm) of Mackerel Surimi Supplemented with Recombinant and Glycosylation Chicken Cystatins Expressed from *P. pastoris* X-33[a]

inhibitory activity (units)	recombinant cystatin		glycocystatin	
	breaking force (g)	deformation (mm)	breaking force (g)	deformation (mm)
0	277 ± 15.9^{a}	6.0 ± 0.31^{a}	277 ± 15.9^{a}	6.0 ± 0.31^{a}
1.0	355 ± 15.2^{b}	7.1 ± 0.36^{b}	344 ± 11.3^{b}	6.3 ± 0.20^{a}
2.0	432 ± 14.5^{c}	8.2 ± 0.42^{c}	380 ± 17.4^{c}	7.0 ± 0.24^{b}
4.0	$480 \pm 29.1^{c,d}$	8.9 ± 0.59^{c}	524 ± 22.9^{d}	8.6 ± 0.33^{c}
8.0	501 ± 27.5^{d}	9.1 ± 0.39^{c}	668 ± 28.4^{e}	10.2 ± 0.44^{d}
12.0	507 ± 17.2^{d}	9.0 ± 0.26^{c}	631 ± 37.1^{e}	10.0 ± 0.31^{d}

[a] Crude recombinant and glycosylation chicken cystatins obtained from culture broth of recombinant *P. pastoris* X-33 were dialyzed against 20 mM phosphate buffer (pH 7.0). [b] Values are means of 10 determinations; values bearing unlike subscripts (a–e) in the same column are significantly difference ($p < 0.05$).

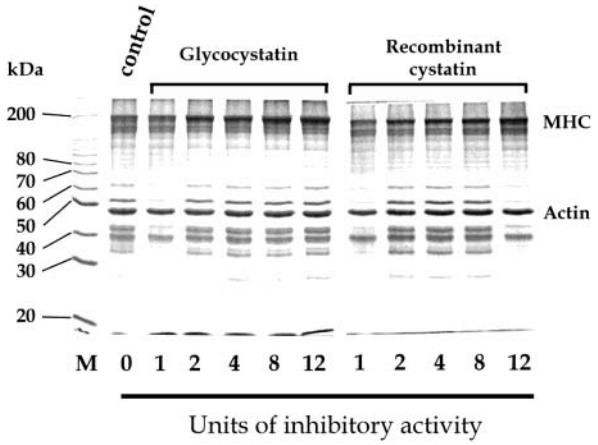

Figure 5. SDS–PAGE analysis of mackerel surimi proteins. Various treatment mackerel surimi gels were incubated in a 95 °C water bath for 10 min, and the dissolved proteins were electrophoretically run on a 10% SDS–PAGE. Lane M, protein marker of a 10 kDa ladder.

deformation of gels reached plateau values, respectively. Despite the additional amount of glycocystatin, which was 2-fold that of recombinant, and the 40 and 15% increases in breaking force and deformation excess, the maximum level of recombinant cystatin gel was also achieved. Nakamura et al. (*29*) reported a similar phenomenon that glycosylated cystatin C markedly improved the gel strength of roe-herring surimi, which was 2.5-fold that of unglycosylated type. As indicated in **Figure 5**, the disintegration on MHC of 1 unit of both cystatin gels was obviously observed in addition to the control gel, whereas the actin in gel-softening surimi gels still remained intact. Previous studies had pointed out that the catheptic cysteine proteinases such as cathepsins B, L, and L-like could hydrolyze MHC and lead to gel softening (*1*, *3*, *4*, *7*). The degradation of MHC seems to be progressively inhibited accompanying the raise addition of either cystatins. On the other hand, the change in surimi proteins was consistent with gel properties determined by the punch test. Because the gel softening that occurred in mackerel surimi was mostly caused by the hydrolysis of catheptic cysteine proteinases, the cystatin could be practically utilized for inhibiting them (*7*). However, there were other species of protease rather than cathepsin left in mackerel mince that could reduce the inhibitory ability of cystatin. The glycosylation on chicken cystatin therefore enhanced the structural stability as well as the preventive effect on gel softening of fish mince.

In conclusion, *P. pastoris* X-33 carrying a site direction mutant (N-Q-^{108}I→N-Q-^{108}T) chicken cystatin secretion ex-

pressed a moderate glycosylation modification cystatin with 50 DP of mannose. In addition to freezing stability (*12*), the resistance upon heating, pH shift, and the susceptibility against protease was enhanced as well. Furthermore, the inhibitory activity almost remained in glycocystatin as compared to that of the unglycosylation form. As reported previously, the culture broth of *P. pastoris* transformant secretion expressed more than half of the recombinant and glycosylation cystatins. This makes the application of crude samples on fish mince simplified. In the study, the addition of glycocystatin further increases the gel texture of mackerel surimi by simultaneously enhancing the stability.

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