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Application of polymannosylated cystatin to surimi from roe-herring to prevent gel weakening

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Abstract A recombinant glycosylated cystatin with a polymannosyl chain was added to roe-herring surimi for preventing gel weakening due to autolysis during cooking. Proteolysis of myosin heavy chain in the surimi was effectively suppressed while cooking at 90°C for 20 min after preincubation at 40°C for 30 min. The glycosylation of cystatin improved the stability against heating as well as proteolysis by cathepsin D. This process markedly improved the texture of the cooked surimi gel with gel strength 2.5 times that of unglycosylated control cystatin.

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Key words: Polymannosylated cystatin; Molecular stability; Surimi; Heat-induced gel; Roe-herring

1. Introduction

Surimi, a water-leached, cryostabilized fish mince muscle, is a raw material for making gelled seafood products, such as kamaboko and imitation crabmeat. An increase in the demand for surimi products has increased the interest among researchers in the utilization of underutilized fish, such as roe-herring and late-run salmon. As a result, proteinase inhibitors are recently calling attention to make good surimi-based products from the underutilized fish because most of them contain endogenous muscle proteinases, which weaken the gel strength by degrading myosin heavy chain (MHC) during cooking of surimi [1,2]. Since major lysosomal proteinases in animal cells are papain-like enzymes [3,4], cystatin, an inhibitor of sulfhydryl proteinases, would be a useful ingredient to achieve this purpose. However, cystatin C was inactivated by hydrolysis of hydrophobic sites with cathepsin D, an aspartyl proteinase [5]. Therefore, the improvement in conformational stability of this inhibitor is required to avoid the risk of degradation during the preparation of surimi from underutilized fish.

Olden et al. [6] and Gu et al. [7] reported that carbohydrate moieties contributed to the protection of polypeptide chains of glycoproteins from proteolysis. We propose that the site-specific glycosylation of proteins occurred in yeast is a new approach to enhance their molecular stability against heating and proteolysis [8]. Recently, we were successful in obtaining a stable cystatin glycosylated with polymannosyl chains using two yeast expression systems [9]. The chain-length of mannosylation of cystatin derived from *Pichia pastoris* was shorter and more homogeneous than that from *Saccharomyces cerevisiae*, and this 30-kDa glycosylated cystatin had a high papain-inhibiting activity [9].

In this study, the polymannosylated cystatin from *P. pastoris* was used to make surimi products with high gel strength from roe-herring. The present paper also deals with the possible application of a recombinant protein derived from yeast in the seafood industry.

2. Materials and methods

2.1. Materials

Polymannosylated cystatin with a molecular mass of 30 kDa and unglycosylated cystatin were obtained from *Pichia pastoris* GS115 (his 4) carrying a mouse cystatin C gene as described [9]. Papain (type III), cathepsin D, pepstatin A and *N*-α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade or biochemical use.

2.2. Molecular stability tests

Heat inactivation of recombinant cystatins was monitored by heating cystatin at various temperatures for 30 min. Inhibitor samples at 0.1 mg/ml in 50 mM Tris-HCl buffer (pH 7.5) were incubated in a sealed vial to avoid evaporation. After heating, aliquots were removed from the solution in the vial and chilled in icewater. The residual activity of the heated solutions was compared to that of the sample before heating, and expressed as its percentage.

The papain-inhibiting activity was determined by measuring the inhibitory activity using BAPNA as substrate [10]. Susceptibility of recombinant cystatins to cathepsin D was measured to determine the conformational stability of polymannosyl cystatin. Five hundred μl of a 0.1 mg/ml solution of cystatin was incubated with an equal volume of 0.5 μM cathepsin D at 37°C. After a pre-set time up to 40 min, 100-μl samples were removed and mixed with 1 μM pepstatin A to terminate the enzymatic activity of cathepsin D [11]. Residual papain-inhibiting activity determined using BAPNA was expressed as percentage of the initial activity in a similar manner as in the case of the heat stability test.

2.3. Preparation of surimi and heat-induced gel

Fresh roe-herring was obtained from a local fishery and fillets prepared from it were stored at −80°C. Herring surimi was prepared from the frozen fillets according to the procedure of Toyota et al. [12]. The surimi was ground with 3% NaCl and 10 μg/g recombinant cystatins at 4°C for 10 min. After the surimi paste was stuffed into a 1.5-ml plastic microtube, the paste was centrifuged at 5000×*g* for 5 min to remove trapped air bubbles. Surimi gel was prepared by heating at 90°C for 20 min after preincubation at 40°C for 30 min. The microtube containing surimi gel was then immediately chilled into an ice bath. The bottom of the tube was cut, and the surimi gel was pressed out into a 1.5-cm syringe with a 0.9-cm diameter. The resultant cylindrical gel was cut into 10-mm slices.

2.4. Measurement of gel strength

Gel strength of the heat-induced surimi was measured by punch test using TA-XT2 texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a 5-mm cylinder plunger. The breaking strength represented as the load value (N) at the breaking point when the surimi gel was compressed at a crosshead speed of 30 mm/min. Gel strength was determined by multiplying the breaking strength (N) and deformation (mm) [13]. Data obtained from triplicate experiments were used to calculate the averages and standard deviations.

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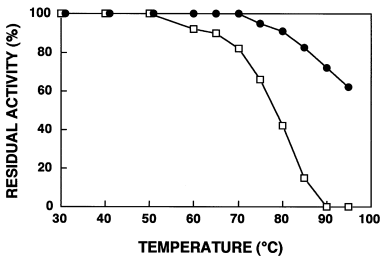

Fig. 1. Heat stability of recombinant cystatins. Recombinant cystatins were incubated in pH 7.5 buffer at 0.1 mg/ml protein concentration at various temperatures for 30 min. The residual activity of each incubated sample was determined by measuring papain-inhibiting activity and compared with that of the corresponding sample without heating. □, unglycosylated cystatin; ●, polymannosylated cystatin. Data shown are the averages from triplicate experiments.

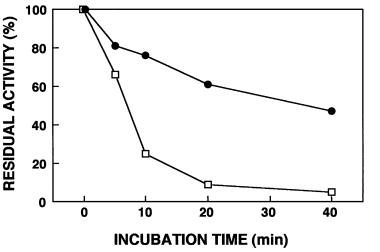

Fig. 2. Susceptibility of recombinant cystatins to cathepsin D. 0.1 mg/ml recombinant cystatin was incubated with an equal volume of cathepsin D (0.5 μM) at 37°C. At a pre-set time, 100-μl samples were removed and added to 1 μM pepstatin A to inhibit the enzymatic activity of cathepsin D. Papain-inhibiting activity of the solution was measured using BAPNA as substrate and compared to that of the corresponding sample without cathepsin D treatment. □, unglycosylated cystatin; ●, polymannosylated cystatin. Data shown are the averages from triplicate experiments.

2.5. SDS-polyacrylamide gel electrophoresis

Gel electrophoresis was carried out according to Laemmli [14] using 10% acrylamide separating gel and 3% stacking gel containing 1% SDS. Ten mg of surimi sample in 1 ml 25-mM Tris-HCl buffer (pH 8.0) containing 10% SDS, 8 M urea, and 0.1% β-mercaptoethanol was incubated at 37°C overnight. The mixture was centrifuged at 17 500×*g* for 15 min to remove undissolved pieces after vigorous agitation using a vortex mixer (Vortex 2 GenieTM, Bohemia, NY). An aliquot taken from the middle layer of the supernatant was heated at 100°C for 5 min in Tris-glycine buffer (pH 8.8) containing 1% SDS and 1% β-mercaptoethanol. Electrophoresis was carried out at a constant current of 10 mA for 5 h using an electrophoretic buffer of Tris-glycine containing 0.1% SDS. After the electrophoresis was completed, the gel sheet was stained with 0.025% Coomassie Brilliant Blue R-250.

3. Results and discussion

3.1. Molecular stability of polymannosylated cystatin

The stabilities against heating and cathepsin D proteolysis were investigated. Fig. 1 shows the heat resistance of polymannosylated cystatin compared with unglycosylated control when 0.1 mg/ml protein solution was heated at different temperatures for 30 min. The polymannosylated cystatin was found to be appreciably more thermostable than the unglycosylated control. Heating the polymannosylated protein at 95°C for 30 min resulted in less than 40% reduction of the papain-inhibiting activity. Although it is reported that cystatin C is resistant to heating due to the tightly packed conformation [15], further enhancement of the thermostability was observed in the polymannosylated cystatin.

Cathepsin D, a typical lysosomal aspartyl proteinase, is widely distributed in all mammalian tissues [4]. Pepstatin A, an inhibitor of aspartyl proteinases [11], was used to avoid further digestion of recombinant cystatins by cathepsin D after a certain incubation time. As shown in Fig. 2, the papain-inhibiting activity was better maintained in the polymannosylated cystatin than in the unglycosylated control. The papain-inhibiting activity of the polymannosylated 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. It is assumed that the polymannosylation of cystatin can enhance its conformational stability against cathepsin D.

The polysaccharide chain could disturb the physical accessibility of cathepsin D to the proteolytic cleavage sites in the polymannosylated cystatin. It is generally agreed that cathepsins B, D, H, and L are major lysosomal proteinases participating to intracellular protein breakdown in the mammalian cells [3,4]. Endogenous inhibitors for cathepsins B, H, L belonging to the sulfhydryl cathepsin group were found in various animal tissues. Cathepsin D may play an important role in the degradation of proteins through limited proteolysis [3,4]. Recently, it was reported that cathepsin D was responsible for the regulation of sulfhydryl proteinase activity by inactivating cystatin and kininogen [5]. Reduced activity of sulfhydryl-proteinase inhibitor was found in the white muscle of chum salmon with enhanced activity of lysosomal proteinases, during spawning migration [2]. Since it is known that sulfhydryl-proteinase inhibitors are the most active agents compared with other seryl- and aspartyl-proteinase inhibitors [16,17], improvement in the conformational stability against cathepsin D should play a key role in the maintenance of the gel strength of herring surimi gel.

3.2. Effect of polymannosylated cystatin on gel strength of heat-induced surimi gel

The polymannosylated cystatin was added to roe-herring surimi prior to the preparation of a heat-induced surimi gel. Fig. 3 shows the gel strength of surimi gel containing 10 μg recombinant cystatins per g protein. Without inhibitor, the herring surimi did not form a gel strong enough to be measured by the punch test. Since sufficient proteinases remained

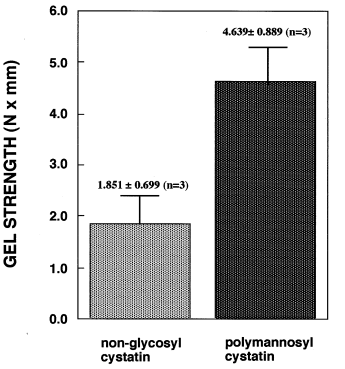

Fig. 3. Gel strength of heat-induced herring surimi gels. Recombinant cystatins were added to the surimi at 10 μg/g protein concentration and the breaking strength of the gels was measured by the punch test (see Section 2 for experimental details).

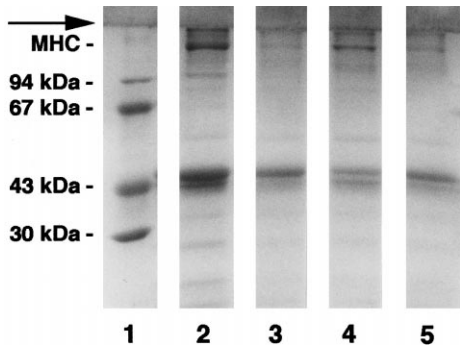

Fig. 4. SDS-PAGE patterns of surimi proteins in heat-induced gels. Electrophoresis was carried out at a constant current of 10 mA for 5 h in Tris-glycine buffer (pH 8.8) containing 0.1% SDS. The gel sheets were stained with Coomassie Brilliant Blue. Arrows indicate the position of the boundary between the stacking (upper) and separating (lower) gels. Lane 1, molecular weight markers; lane 2, herring surimi prior to gel formation; lane 3, heat-induced surimi gel without cystatin; lane 4, heat-induced surimi gel with polymannosylated cystatin; lane 5, heat-induced surimi gel with unglycosylated cystatin; MHC, myosin heavy chain.

in the herring surimi, the gel formation was interfered by proteolysis of MHC. The addition of recombinant cystatins to surimi was effective in increasing the punch force values (Fig. 3). This result indicates that sulfhydryl proteinase was involved in the textural degradation during cooking. The herring surimi gel with the polymannosylated cystatin revealed significantly higher gel strength than that with the unglycosylated cystatin. The effect of the polymannosylated cystatin on the gel strength of surimi gel was 2.5 times that of the unglycosylated cystatin. That the gel strength increased by adding recombinant cystatin may be due to the conformational stability against heating and proteolysis through polymannosylation of cystatin.

Evidence of enhanced gel formation using polymannosylated cystatin was shown in SDS-polyacrylamide gel electrophoresis of roe-herring surimi proteins. As indicated in Fig. 4, a preventive effect of recombinant cystatins on gel weakening was observed in the surimi gels. Recombinant cystatins inhibited the degradation of MHC in herring surimi during cooking. No significant decomposition of both MHC (220 kDa) and actin (45 kDa) was observed in the surimi gel prepared by adding polymannosylated cystatin (lane 4).

In conclusion, polymannosylated cystatin was effective in maintaining good gelling properties in roe-herring surimi because of its inhibitory activity for sulfhydryl proteinases by enhancing molecular stability. Surimi gel with a good texture can be prepared from roe-herring by adding polymannosylated cystatin. Polymannosylated cystatin may also be useful for kamaboko-making from surimi derived from other fish species.

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References

[1] An, H., Weerasinghe, V., Seymour, T.A. and Morrissey, M.T. (1994) J. Food Sci. 59, 1013–1017.
[2] Yamashita, M. and Konagaya, S. (1990) Comp. Biochem. Physiol. 95B, 149–152.
[3] Turk, V. and Bode, W. (1991) FEBS Lett. 285, 213–219.
[4] Kirschke, H. and Barrett, A.J. (1987) in: Lysosomes –Their Role in Protein Breakdown (Glaumann, H. and Ballard, F.J., Eds.) pp. 193–238, Academic Press, London.
[5] Lenarčič, I.C.B., Krašovec, M., Ritonja, A., Olafsson, I. and Turk, V. (1991) FEBS Lett. 280, 211–215.
[6] Olden, K., Bernet, B.A., Humphries, M.J., Yeo, T.-K., Yeo, K.-T., White, S.L., Newton, S.A., Bauer, H.C. and Parent, J.B. (1985) Trends Biochem. Sci. 10, 78–82.
[7] Gu, J., Matsuda, T., Nakamura, R., Ishiguro, H., Ohkubo, I., Sasaki, M. and Takahashi, N. (1989) J. Biochem. 106, 66–70.
[8] Nakamura, S., Takasaki, H., Kobayashi, K. and Kato, A. (1993) J. Biol. Chem. 268, 12706–12712.
[9] Nakamura, S., Ogawa, M. and Nakai, S. (1998) J. Agric. Food Chem. (submitted).
[10] Arnon, R. (1970) Methods Enzymol. 19, 226–244.
[11] Rich, D.H., Bernatowicz, M.S., Agarwal, N.S., Kawai, M. and Salituro, F.G. (1985) Biochemistry 24, 3165–3173.
[12] Toyoda, K., Kimura, I., Fujita, T., Noguchi, S.F. and Lee, C.M. (1992) in: Surimi Technology (Lanier, T.C. and Lee, C.M., Eds.) pp. 79–81, Marcel Dekker, New York.
[13] Lanier, T.C. (1992) in: Surimi Technology (Lanier, T.C. and Lee, C.M., Eds.) pp. 123–163, Marcel Dekker, New York.
[14] Laemmli, U.K. (1970) Nature 227, 680–685.
[15] Hall, A., Håkansson, K., Mason, R.W., Grubb, A. and Abrahamson, M. (1995) J. Biol. Chem. 270, 5115–5121.
[16] Saeki, H., Iseya, Z., Sugiura, S. and Seki, N. (1995) J. Food Sci. 60, 917–928.
[17] Yamashita, M., Henmi, H., Ueda, T., Obara, M., Taro, T., Nishioka, F. and Konagaya, S. (1996) Nippon Suisan Gakkaishi 62, 934–938.