Surimi Enzymology and Biotechnology

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Introduction

- Surimi made from water-washed fish mince and mixed with cryoprotectants has a unique ability to form elastic gels through the interaction of myosin molecules
- Gel-forming of myosin occurs at two stages during low temperature setting (0~45°C) and high temperature (90°C) heating

- Low temperature setting is responsible for the polymerization of myosin heavy chain (MHC) association with the actions of TGase and with the oxidation of SH groups.
- However, gel disintegration frequently occurs during heating process.

Gelation or Suwari

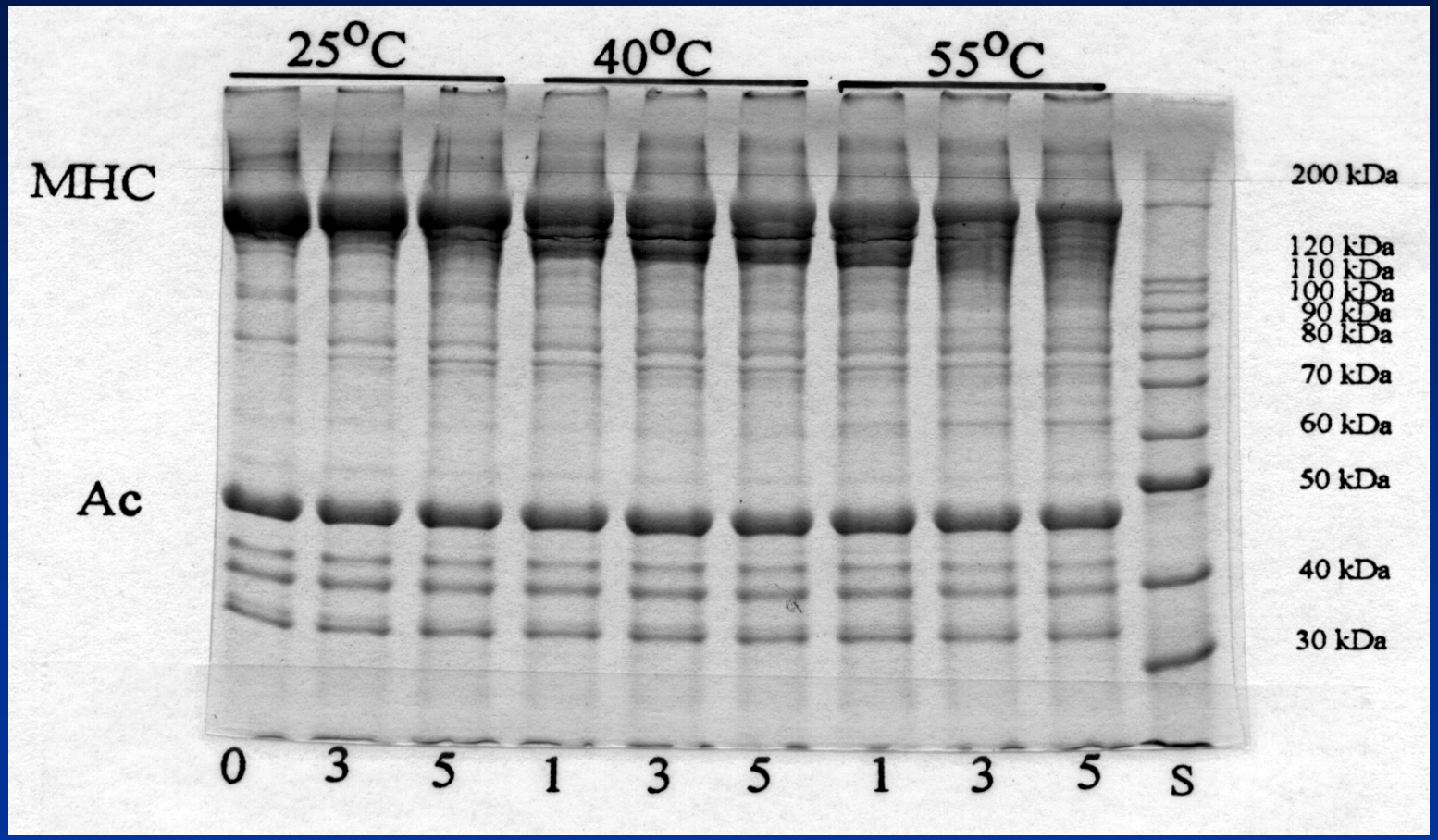
- Surimi gels are a three dimensional protein network formed mainly by actomyosin.
- The gelation of fish actomyosin took place in two stages, at 30-45°C and 50-80°C observed by DSC.

- It is thus proposed that the development of gel elasticity at the 1st stage is due to the interactions between the tails and the 2nd stage due to the interactions among the heads of myosin.

- And the myosin generally gelled by losing its α-helical structure due to heating and then by increasing turbidity due to intermolecular association, and finally forms a rigid structure which is stabilized by covalent disulfide bonds and non-covalent interactions.

- These phenomena suggest that the setting of surimi is initiated by the unfolding of α-helix and propagated by the formation of stabilizing covalent disulfide bonds and non-covalent interactions

Softening (Modori)



SDS-PAGE of the protein degradation of actomyosin extracted from mackerel dorsal muscle incubated at 25 °C , 40 °C and 55 °C , pH 6.5 for 1～5 hr

S：standard proteins ; MHC：myosin heavy chain ; Ac：actin.

- Besides the temperature effects on the conformation of myofibrillar proteins, it can activate endogenous enzymes naturally occurring in fish muscle
- Fish muscles from various species showed similar reactions to temperature: a structure-setting reaction for gelation below 40°C (low temperature setting) and a structure disintegration reaction at 50 - 70°C (modori)

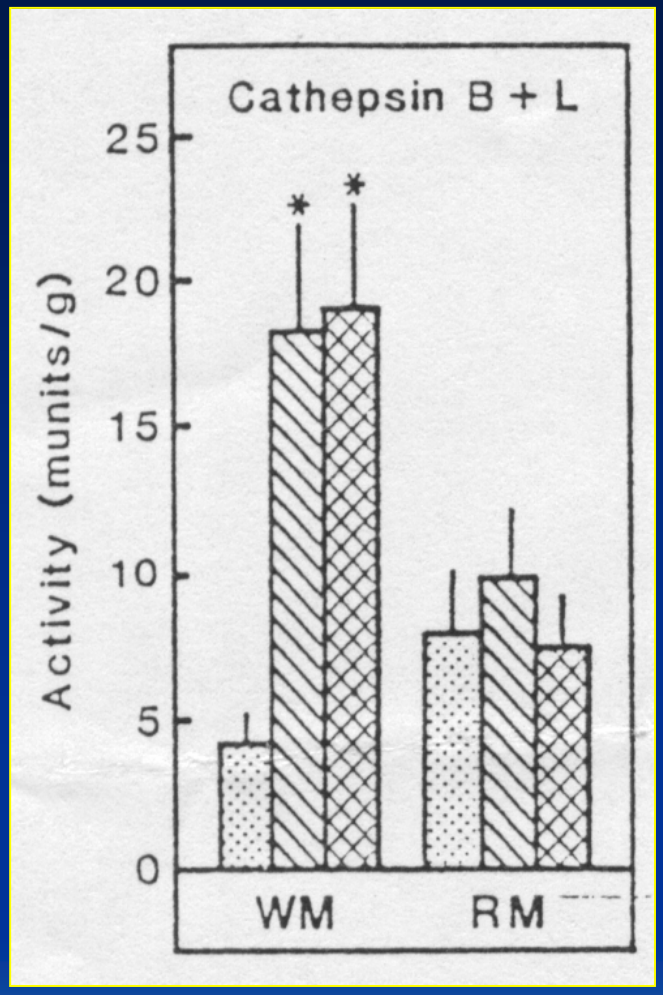
- The most active proteinases in fish muscle which can soften the surimi gels vary with species, but are generally categorized into two major groups: cathepsins and heat-stable alkaline proteinases
- High levels of cathepsins B, H, L, and L-like, have been observed in Pacific whiting, arrowtooth flounder, chum salmon and mackerel

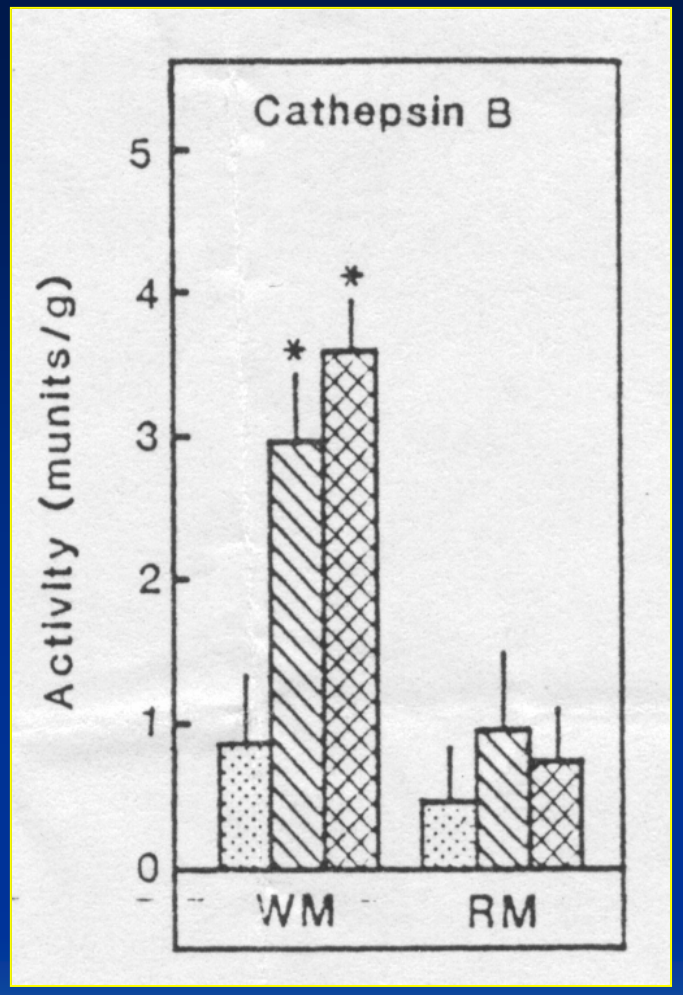
- Cathepsins B, L and L-like have high affinity for myosin, and moderate for troponins and actin
- They can not be completely removed by leaching during surimi processing

Catheptic activity in certain fish and mammalian muscle

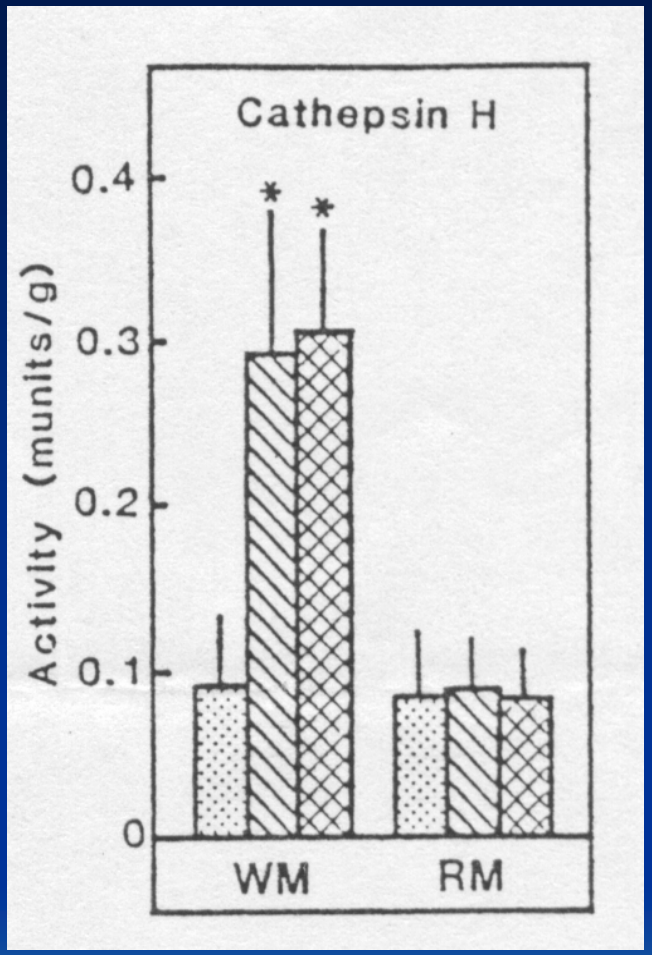
Source	Catheptic activity (Tyrosine Units)
Fish	
Carp (*Cyprinus carpio*)	35
Mackerel (*Rastrelliger kanagurta*)	140
Cod (*Gadus morhua*)	30
Herring (*Clupea harengus*)	41
Trout (*Salmo gairdneri*)	50
Mammals	
Pigs	7
Cattle	8
Rabbits	5

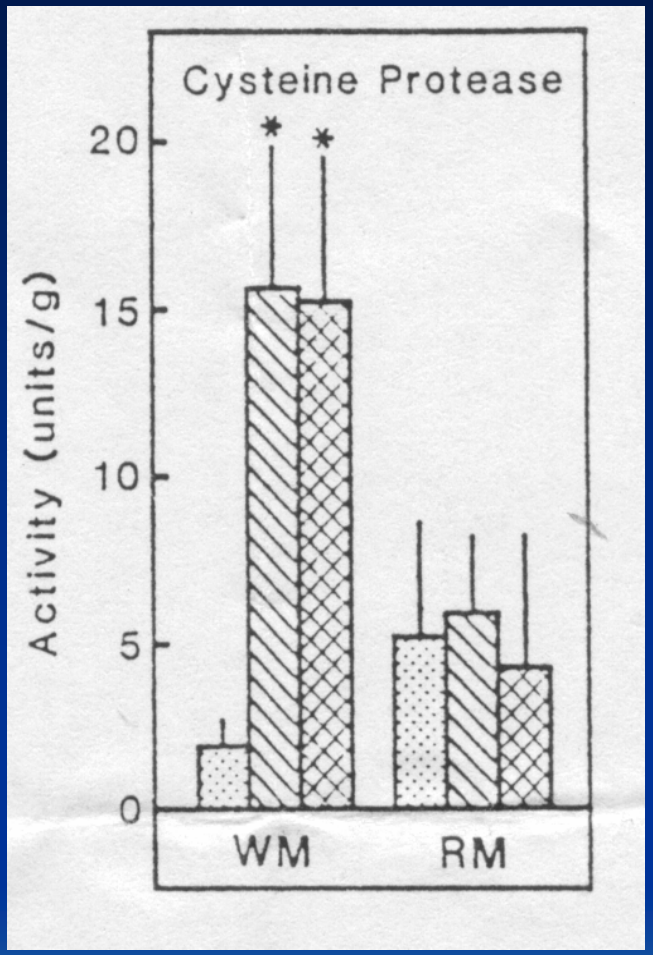
(Source: Mukundan et al., 1985)



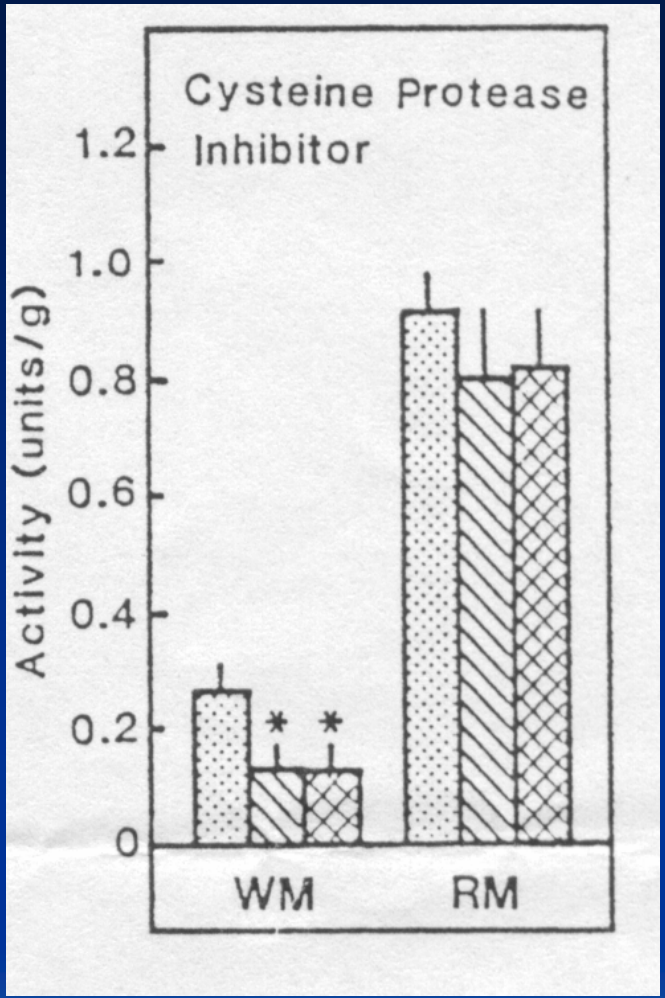


Enzyme activities in the white muscle (WM) and the red muscle (RM) of chum salmon





Enzyme activities in the white muscle (WM) and the red muscle (RM) of chum salmon



Enzyme activities in the white muscle (WM) and the red muscle (RM) of chum salmon

The Questions are:

- How much are these proteinases left after surimi processing?
- Do the freezing and subsequent storage affect their activities?
- Do the cryoprotectants affect their activities?

Cathepsin B and L activity remaining in mackerel meat after each processing step

Process	Enzyme activity (units/g)	Relative activity (%)
Mincing	6.02 ± 0.11^{a}	100.00^{a}
Leaching	5.23 ± 0.12^{b}	86.88^{b}
NaCl-grinding	4.07 ± 0.09^{c}	67.61^{c}

a-c Means of three determinations, expressed as mean ± standard deviation. Values in same column with different letters significantly different ($P<0.05$).

(Jiang, et al., 1997)

Cathepsin B and L activity in mackerel surimi during - 40°C storage

	Storage time (wk)					
	Unfrozen	0	2	4	6	8
Activity (Units/g)	6.02±0.08[a]	5.93±0.11[a]	5.73±0.12[a]	5.60±0.09[ab]	5.25±0.11[b]	4.92±0.09[b]
Relative activity (%)	(100.0)	(98.5)	(95.2)	(93.0)	(87.2)	(81.7)

a-b Means of three determinations, expressed as mean ± standard deviation. Values in same row with different letters significantly different ($P<0.05$).

(Jiang, et al., 1997)

Effects of sucrose, sorbitol and polyphosphate on purified cathepsin B activity during frozen storage

Storage time wks at -40°C	Cathepsin B activity (unit/mL) Control	3% sucrose	3% sorbitol	0.2% PP[1]
0	13.66±0.25aA[2]	12.57±0.26bA	12.76±0.15bA	12.64±0.18bA
2	12.39±0.22aB	12.69±0.21aA	12.76±0.15aA	13.02±0.20aA
4	11.58±0.16bBC	12.31±0.17bA	12.00±0.19bAB	13.80±0.09aA
6	11.33±0.24aC	11.97±0.13aAB	11.53±0.21aB	11.77±0.21aB
8	11.27±0.19aC	11.69±0.16aB	11.33±0.25aB	11.60±0.16aB

[1] PP: mixture of 50% Na-polyphosphate and 50% K-pyrophosphate.

[2] means of 3 determinations, expressed as mean±standard deviation. Values the same row with different lowercases significantly different ($P<0.05$). Values in same column with different uppercases significantly different ($P<0.05$).

Effect of cryoprotectants on the activity of purified cathepsin L during 8 weeks storage at -40 °C

Storage week	Cathepsin L activity (units/ml)		
	Control	3 % Sucrose	3 % Sorbitol
0	3.03	3.06	3.06
2	3.86	3.13	3.07
4	3.25	2.92	3.06
6	2.86	2.88	2.91
8	2.78	2.85	2.84

Effect of cryoprotectants on the activity of purified cathepsin L-like during 8 weeks storage at -40 °C

Storage week	Cathepsin L-like activity (units/ml)		
	Control	3 % Sucrose	3 % Sorbitol
0	21.86[a]	22.00	22.00
2	9.62	11.68	11.37
4	7.63	9.24	10.25
6	7.67	8.50	9.94
8	7.25	8.82	9.39

Gel strength of minced mackerel after 2 hr of cathepsins B and L and E-64[1] incubation at 55°C, pH 7.0

Treatments	Hr at 55°C	Breaking force (g)	Deformation (mm)	Gel strength (g.cm)
None	0	630±15a[2]	10.6±0.8a	668a[2]
None	2	426±18c	8.2±0.4b	349c
Cathespin B	2	399±11d	7.9±0.4b	315d
Cathepsin B and E-64	2	537±21b	9.4±0.5a	504b
Cathespins B and L	2	345±12e	7.5±0.3b	259e
Cathepsins B and L and E-64	2	557±16b	9.5±0.5a	529b

E-64: L-trans-epoxysuccinyl-leucylamido-4-(guanidino)butane.

[2] means of 10 determinations, expressed as mean±standard deviation. Values in same column with different letters significantly different ($P<0.05$).

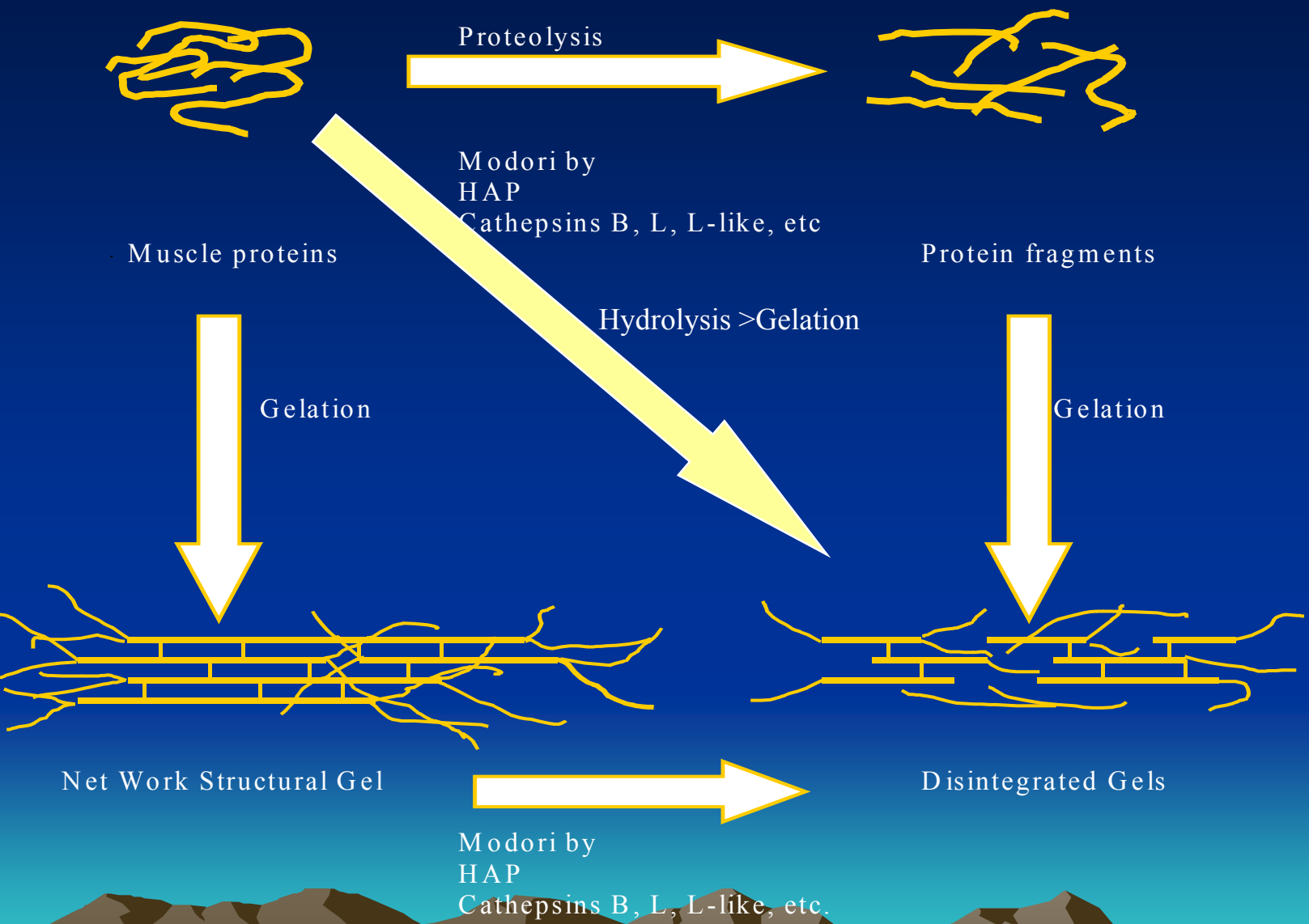


Figure 2. Proposed model of the gelation and disintegration of surimi gels

According to our previous studies,

- reduction of oxidized proteins could enhance the use of frozen stored or processed denatured muscle proteins.
- addition of recombinant inhibitors could inactivate the proteases which could subsequently keep the quality or nutritional value.

- the formation of isopeptide bonds by MTGase could substantially increase the gel properties of surimi-based products or cohesive property of restructured steaks, etc.

Some cases studies on the utilization of MTGase, sulfide reductase, recombinant cystatin (protease inhibitor) in seafood processing

New Approach to Improve the Seafood Processing by Biotechnology

Most of the materials for seafood processing are frozen fish

Characteristics of Frozen Fish

- Loss of functionality due to freezing and subsequent storage such as loss of gel forming ability
- Denaturation due to the oxidation such as protein denaturation and discoloration
- Decrease of the utilization of the frozen fish

Denaturation of Frozen Fish Muscle

Because of the formation of inter- and intra-molecular

- **hydrophobic**
- **hydrophilic interactions**
- **disulfide bonds**

Hypothesis

- The hydrophobic and hydrophilic interactions can be broken by grinding process due to their low bond energy
- The disulfide bonds with high bond energy cannot be broken simply by grinding
- The –SS- may be reduced by the addition of reductants or reductases during grinding process and subsequently recover –SH groups

Table 1. Effect of the Reductants on the Recovery of Reactive-SH Group of the Actomyosin of Freeze-Thawed Mackerel

Additive	Level of addition		
	0.05%	0.08%	0.10%
	recovery (%)		
Mercaptoethanol	71.4%b^{b}B^{c}	83.2%aA	84.8%aA
Cysteine	67.6%bB	82.5%aA	85.6%aA
Tannic acid	58.7%^{c}C	70.7%bB	75.2%aB
Sodium bisulfite	78.2%bA	83.3%aA	85.8%aA
Ascorbic acid	63.5%^{c}C	72.4%bB	77.4%aB

*: The percentage of the reactive-SH group in frozen mackerel was 50.2% relative to that of iced sample. Those of reactive-SH group in treated samples were significantly different from that of untreated frozen muscle ($P<0.01$)

Table 2. Effect of the Reductants on the Extractability Actomyosin (mg/g of meat) of the Freeze-Thawed Mackerel

Additive	Level of addition		
	0.05%	0.08%	0.10%
		recovery (mg/g)	
Mercaptoethanol	168.4C^{b}A^{c}	177.8bB(85%)	189.5aA(90.8%)
Cysteine	159.1cB	167.4bC(80%)	178.6aB(86%)
Tannic acid	144.5cC	158.1bD(76%)	169.6aC(81%)
Sodium bisulfite	172.4bA	188.4aA(90%)	190.2aA(91%)
Ascorbic acid	156.2cB	169.9bC(81%)	174.2aB(84%)

*: The extractability of actomyosin of iced and freeze-thawed mackerel was 208.6 and 136.5 mg/g of meat, respectively

Table 3. Effect of the Reductants on the Quality of Kamaboko prepared from Freeze-Thawed Mackerel

Additive	Level of addition					
	0.05%		0.08%		0.10%	
	G. S.	F. T.	G. S.	F. T.	G. S.	F. T.
Mercaptoethanol	520C[b]A[c]	A	580bB	AA	640aB	AA
Cysteine	535cA	AA	565bB	AA	636aB	AA
Tannic acid	490bB	A	505bC	AA	548aD	AA
Sodium bisulfite	554bA	AA	660aA	AA	685aA	AA
Ascorbic acid	510B	A	565bB	AA	595aC	AA

*: Gel-strength and grade of folding test of the control sample (without reductants) were 450 g. cm and A-AA grade, respectively. G. S. = gel-strength (g. cm); F. T. = grades of folding test. AA: no cracks occurred when folded into semicircle; B: cracks occurred when folded into semicircle; C: broke into two pieces when folded into semicircle.

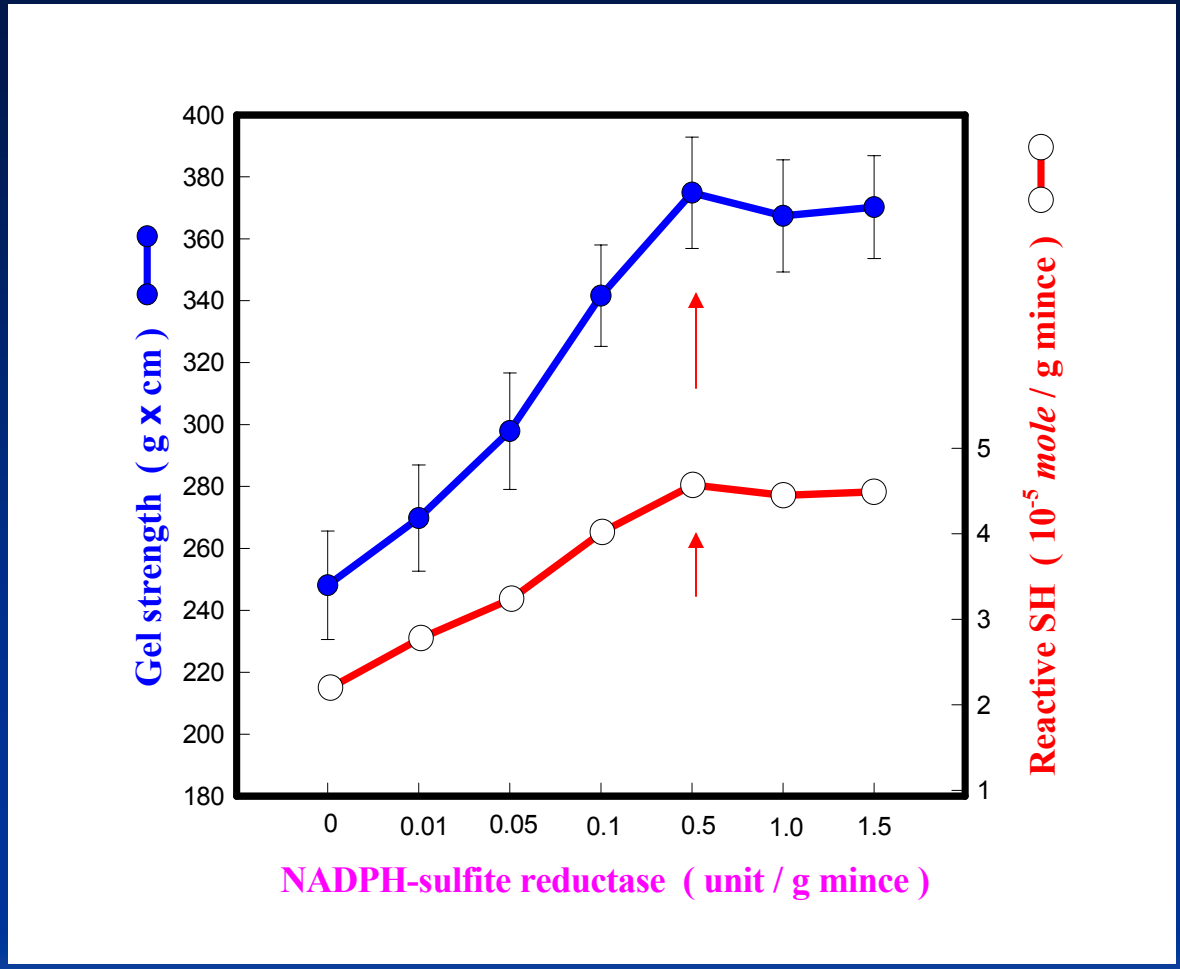


Fig. Effect of NADPH-sulfite reductase amounts added on the reactive sulfhydryl group and gel strength of ozonated mackerel surimi. (○: reactive sulfhydryl group, ● : gel strength)

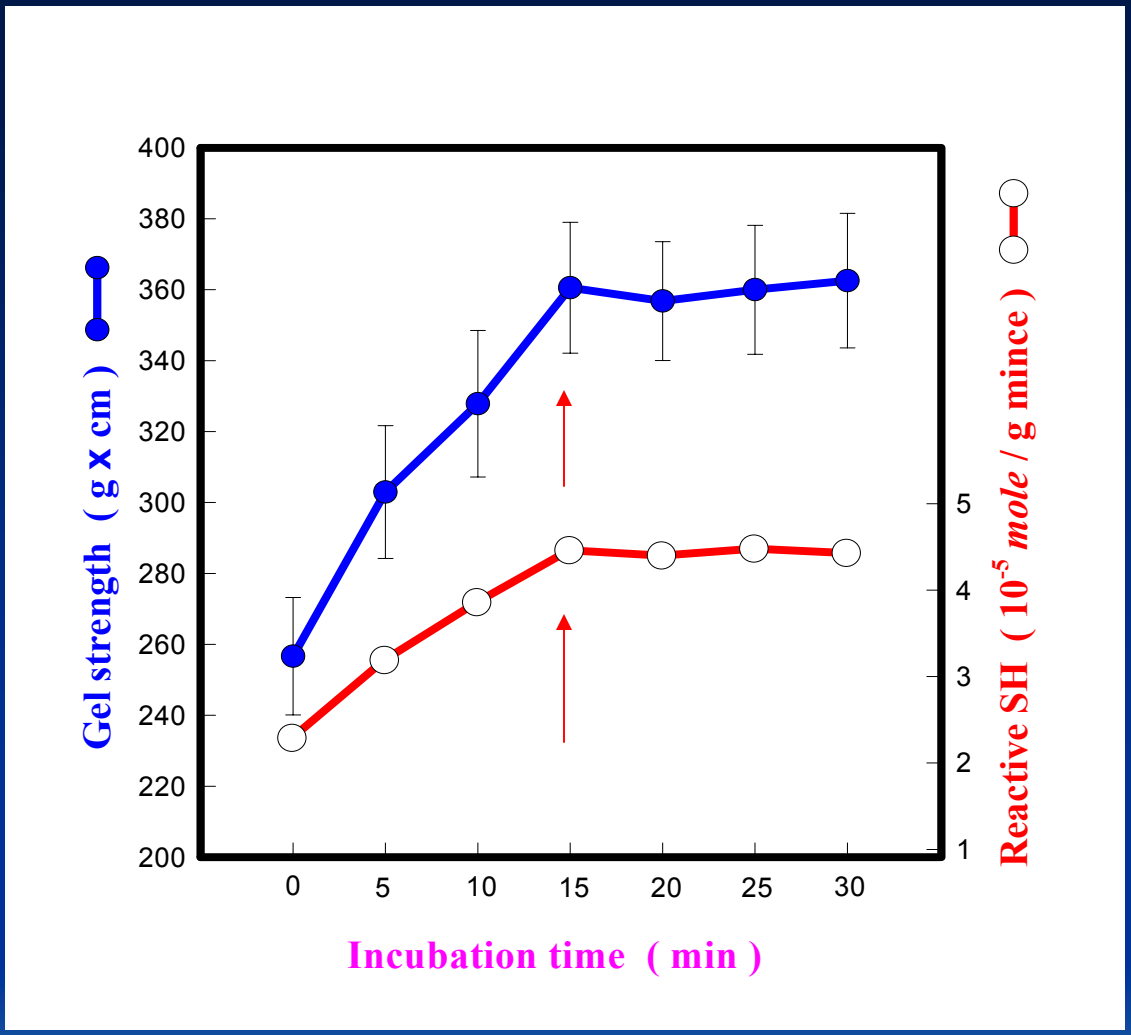


Fig. Effect of incubation time at 25°C on the reactive sulfhydryl group and gel strength of the ozonated mackerel surimi with NADPH-sulfite reductase. (○: reactive sulfhydryl group, ● : gel strength)

From these studies, the addition of reducing agents or NADPH-sulfite reductase could recover the SH groups and consequently increased the gel strength of frozen fish or ozonized fish minces

Production of Proteinase Inhibitors to Improve the Surimi Quality using Biotechnology

The cystatin superfamily

- **Family 1 (the stefins)** →No disulphide bonds
  - Human cystatins A, B
  - Rat cystatins α, β
  - Oryzacystatin I, II
- **Family 2 (the cystatins)** →containing disulphide bonds
  - Human cystatin C, D, SA, SN, SU
  - Chicken cystatin
- **Family 3 (the kininogens)** →containing three copies of family 2 cystatin sequences, larger molecular size
  - L-, H- kininogen (human, rat, bovine)
  - T- kininogen (rat)

Generalizations about cystatins

- *Mr* values are in the range 11kDa - 13.5kDa.
- Amino acid sequence homology.
- **Stable in the temperature range of 80 - 100°C.**
- **Stable in the pH range of pH 2 - pH 12.**
- Reversible, competitive and tight-binding mechanism.
- Inhibit most papain-like proteinases in docking model.
- Inhibit dipeptidyl peptidase I.
- Can not inhibit serine, aspartic or metallo-proteinases.

Inhibition constants for the cathepsins

Ki (nM)	Cathepsin B	Cathepsin H	Cathepsin L
Cystatin A	8.2	0.31	0.05
Cystatin B	73	0.58	0.07
Cystatin C	0.25	0.28	0.008
Cystatin D	>1000	8.5	0.24
Cystatin SN	19	-	-
Chicken cystatin	1.7	0.064	0.019
H-kininogen	600	1.2	-
L-kininogen	400	1.1	-

Total RNA from chicken lung or fish muscle

cDNA

cDNA

Recombinant cDNA for chicken lung or fish cystatin

Recombinant cDNA for Chicken Lung or Fish Cystatin

Transformation & Expression

- Express in Expression Vector
- Transform into *E.Coli* or Yeast
- Optimize the Conditions for Expression

Recombinant cDNA for Chicken Lung or Fish Cystatin

Compare the Sequence and Characters with other Cystatins

Recombinant cDNA for Chicken Lung or Fish Cystatin

Use Fusion Proteins such as thioredoxin, polyhistidine tag, kininogen domain 3 or other stable Proteins to increase the expression or adjust the activity and freezing tolerance, which consequently increase the exploitation of recombinant cystatin

Recombinant cDNA for Chicken Lung or Fish Cystatin

Use site-directed mutagenesis or Error Prone PCR techniques to modify cystatin, which consequently increase the exploitation of recombinant cystatin

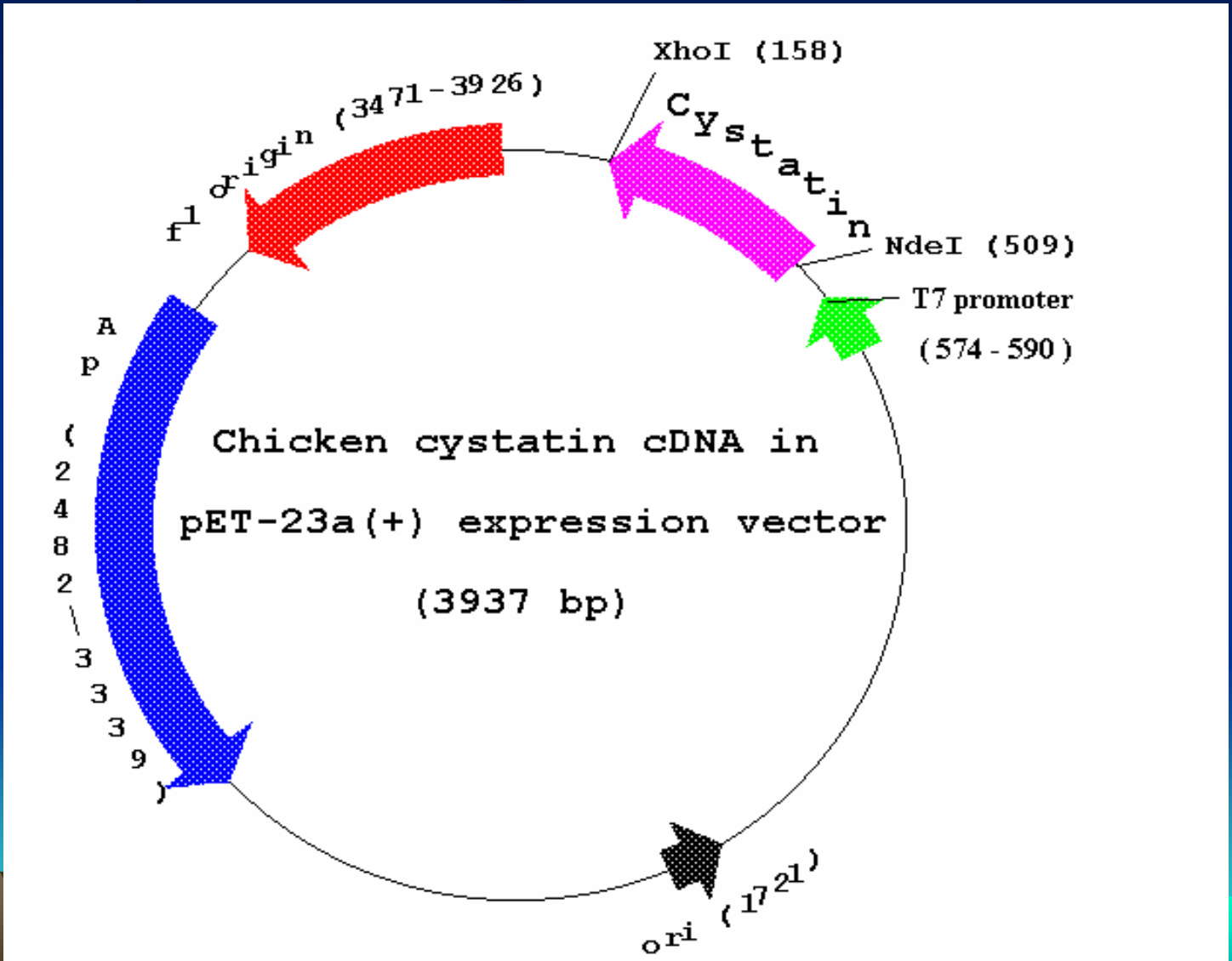
Recombinant Cystatin with high activity and stability

Optimization on amount, processing conditions and method

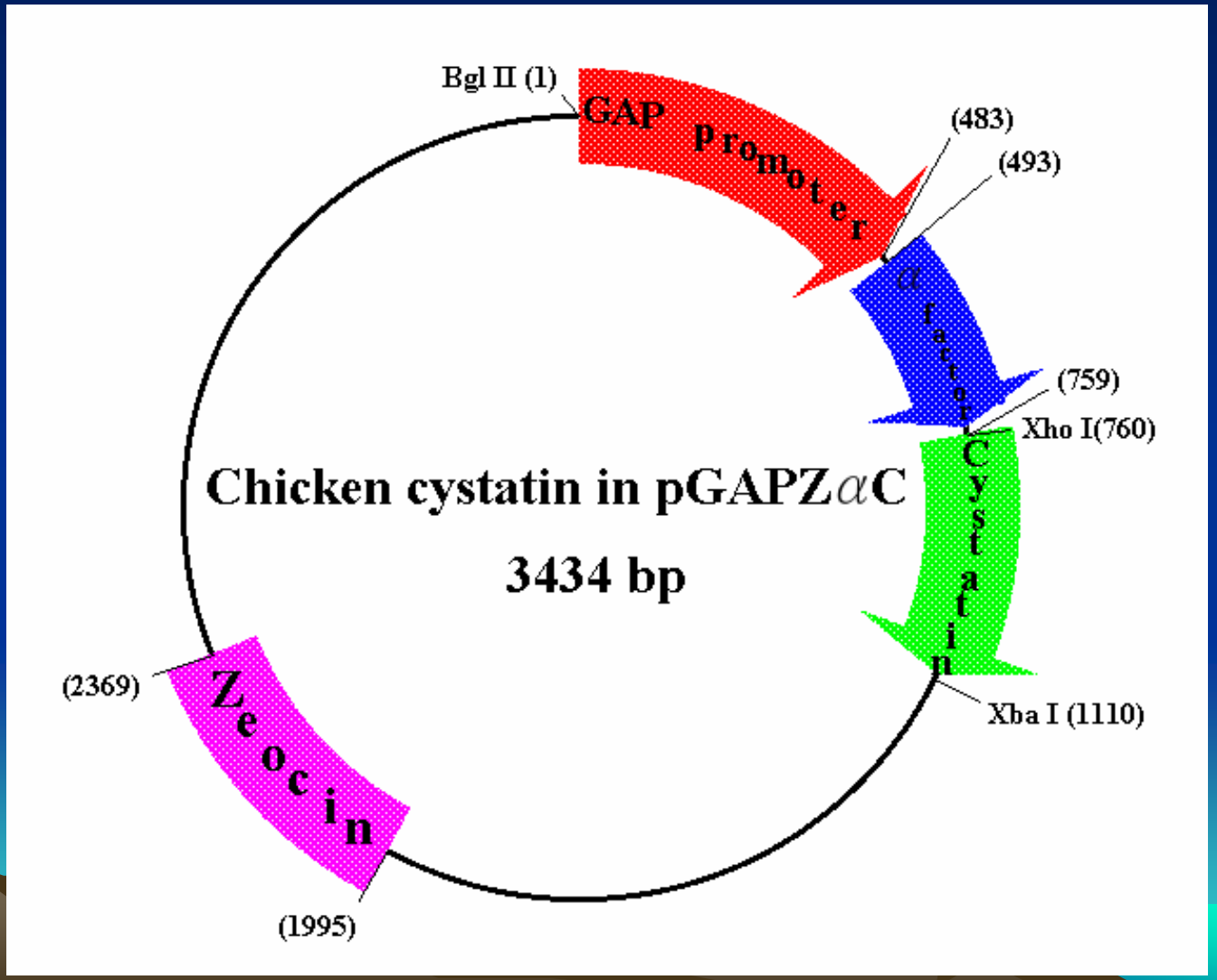
- Apply on the refrigerated, frozen or processed seafoods
- Inhibit the proteolysis of fish muscle proteins
- Prevent the gel softening of surimi-based products

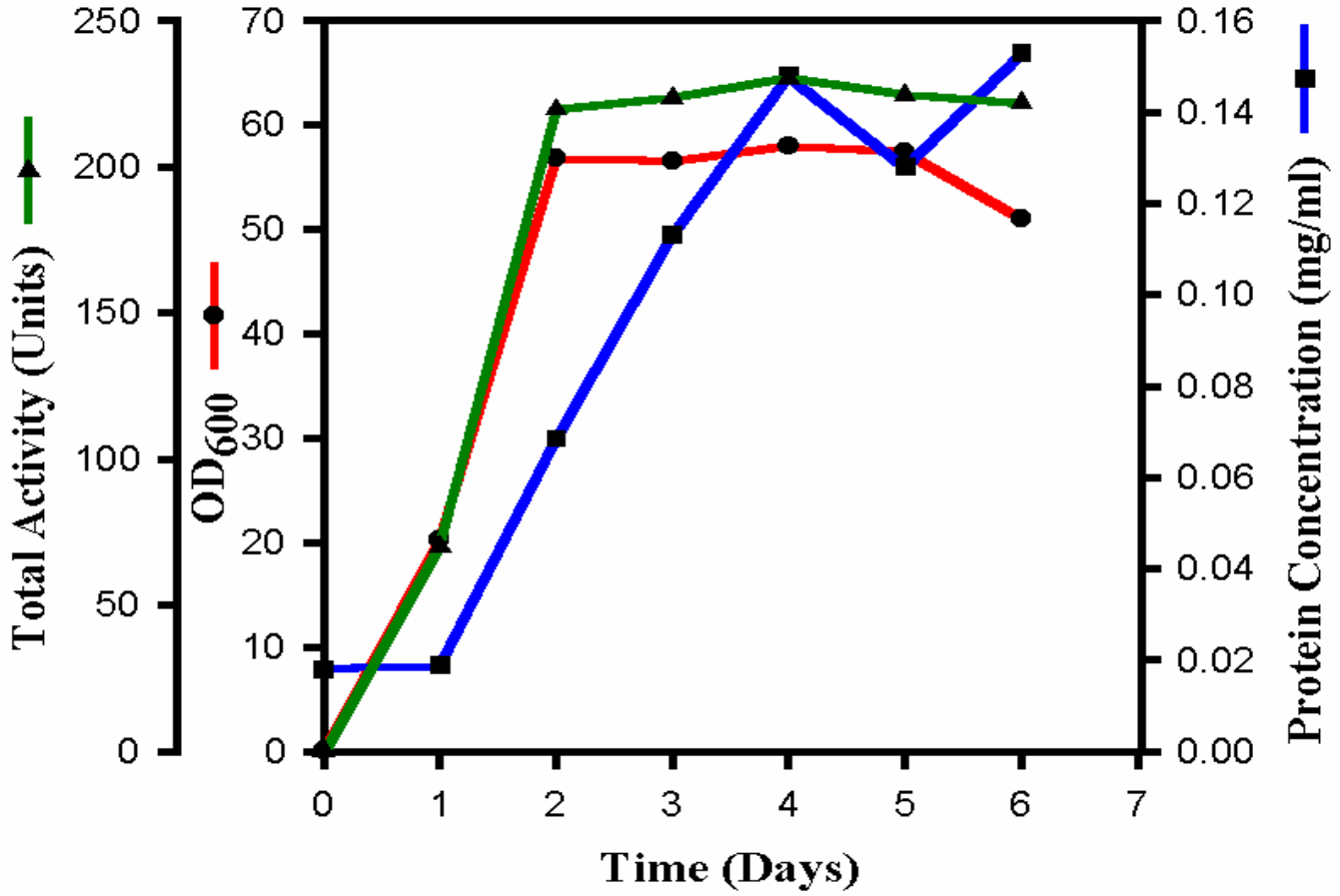
Cloning & Expression of Cystatin in *E. coli* AD494(DE3)pLysS and *Pichia pastoris* X-33

Construction of the pET-23a(+)-cystatin expression vector

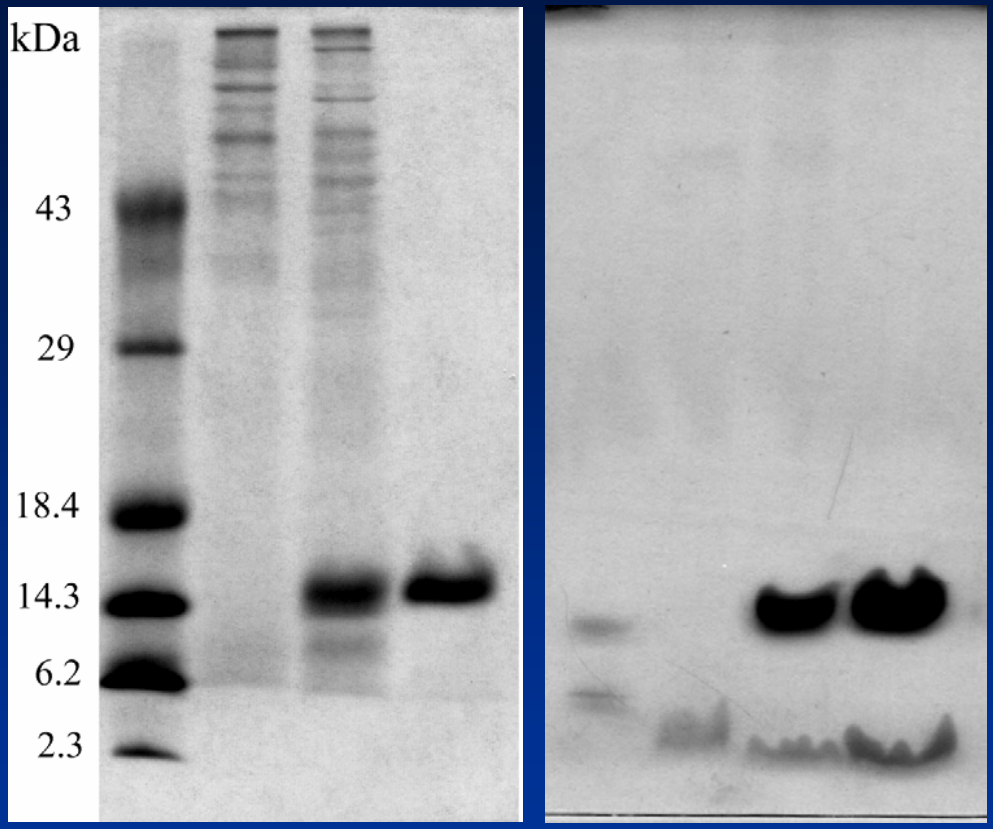


Construction of the pGAPZαC- cystatin expression vector



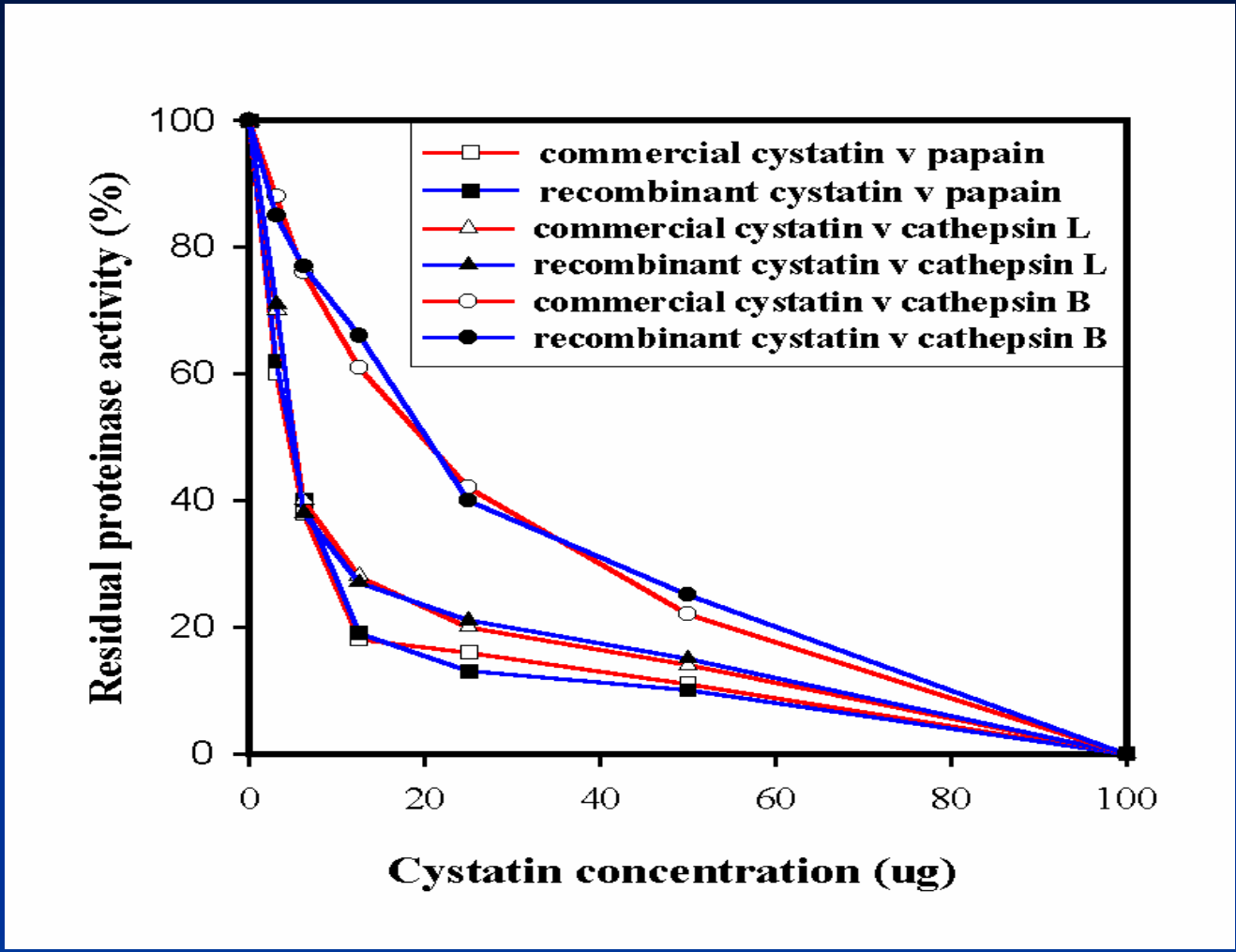


Growth curve of the recombinant *Pichia pastoris*



SDS-PAGE (A) and substrate SDS-PAGE (activity staining, B) of the recombinant chicken cystatin.

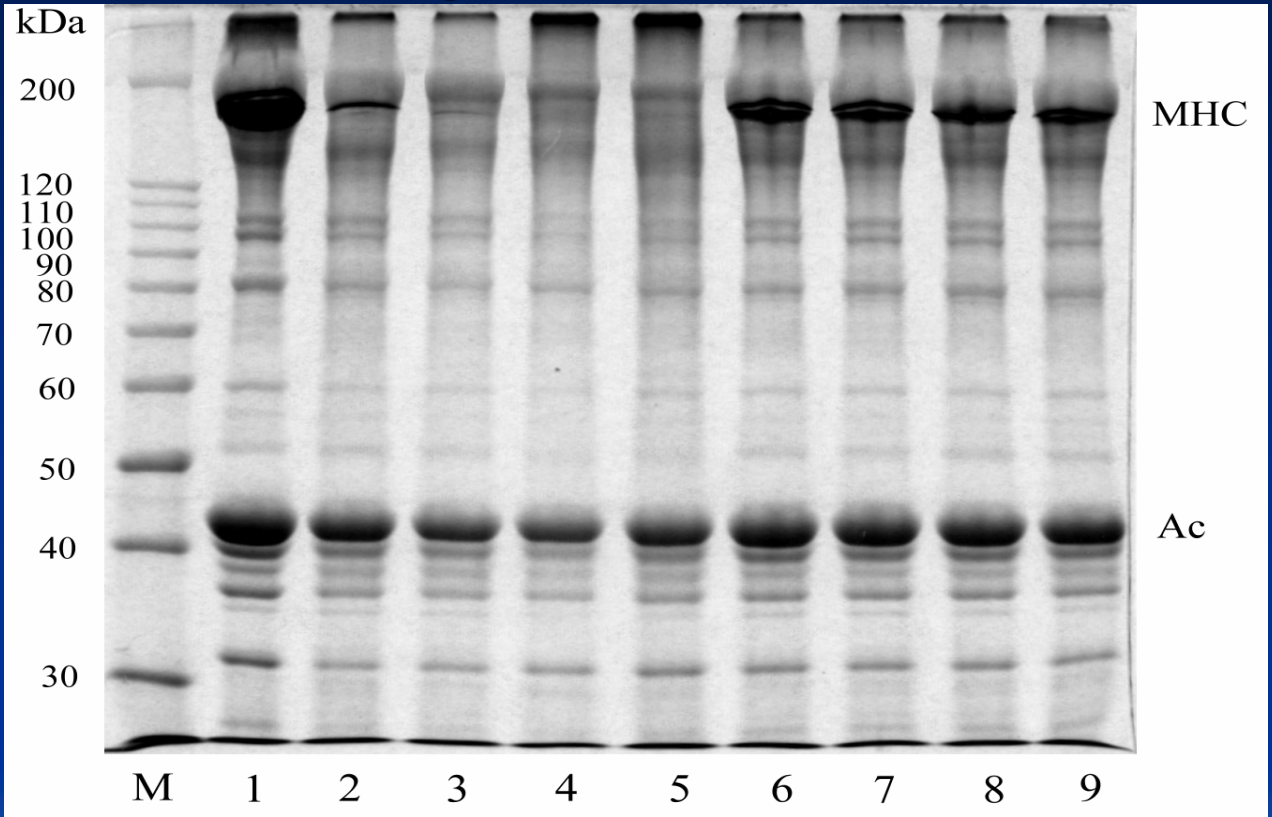
[M: Low MW protein marker; 1: *Pichia pastoris* X-33 (non-transformed with pGAPZαC-cystatin expression vector); 2: Crude recombinant cystatin secreted from *Pichia pastoris* X-33 (transformed with pGAPZαC-cystatin expression vector); 3: purified recombinant cystatin (treated with β-Me); 4,5 and 6 were the electrophoram of substrate (0.1% casein) SDS-PAGE, corresponding to 1, 2 and 3, on SDS-PAGE, respectively. Bands of the unhydrolyzed casein (Coomassie brilliant blue stained) indicate the existence of cystatin.]



Comparison of the inhibition activities of recombinant chicken cystatin and commercial cystatin against papain-like proteinases

(values in this Figure are the means of 3 replicates)

Effect of recombinant chicken cystatin from *Pichia pastoris* X-33 on the inhibition of gel softening of mackerel surimi



M: protein marker; MHC: myosin heavy chain; Ac: actin; Lane 1: heated immediately at 100°C for 30 min; Lanes 2,3, 4 and 5: preincubated at 50°C for 15, 30, 60 and 90 min, respectively, prior to the heating at 100°C for 30 min; Lanes 6, 7, 8 and 9: ground with recombinant cystatin (10units/g) and then preincubated at 50°C for 15, 30, 60 and 90 min, respectively, prior to the heating at 100°C for 30 min. All samples were dissolved in 5 volumes of 62.5 mM Tris-HCl buffer (pH 6.8) containing 2.0% SDS, 8.0 M urea and 5.0% β-Me, and then incubated at 30°C for 24 h.

Effect of the recombinant cystatin from *Pichia pastoris* X-33 on the gel properties of mackerel surimi

Treatment	Incubation time at 50°C (min)	Breaking force (g)	Deformation (mm)	Gel strength (g · cm)
No inhibitor	0	1025±36^{a}	12.5±0.7^{b}	1281±95^{a}
No inhibitor	15	843±34^{c}	11.1±0.8^{c}	936±90^{b}
No inhibitor	30	731±33^{d}	10.6±0.5^{c}	775±52^{c}
No inhibitor	60	720±38^{d}	10.4±0.5^{c}	749±58^{c}
No inhibitor	90	680±47^{e}	9.6±0.8^{d}	653±70^{d}
Recombinant cystatin	0	930±52^{b}	14.2±1.4^{a}	**1321±133^{a}**
Recombinant cystatin	15	906±47^{b}	14.3±0.6^{a}	**1296±77^{a}**
Recombinant cystatin	30	913±41^{b}	14.2±0.3^{a}	**1296±70^{a}**
Recombinant cystatin	60	882±40^{b}	13.5±1.2^{a}	**1191±135^{a}**
Recombinant cystatin	90	858±47^{bc}	13.7±0.9^{a}	**1175±65^{a}**

Values in this table are the means of 8~10 replicates, expressed as mean ± standard deviation. Values with unlike superscripts in the same column differ significantly ($p<0.05$).

Sub-conclusion

- After the chicken cystatin cDNA was cloned into the pET-23a(+) expression vector, transformed into *E. coli* AD494(DE3)pLysS expression host and induced by isopropyl-β-D-thiogalactopyranoside (IPTG), high level of active soluble-form cystatin can be expressed in the cytoplasm of the cystatin-transformed *E. coli*.

- The recombinant chicken cystatin can be purified to electrophoretic homogeneity by a simple and rapid method involving heat treatment and Sephacryl S-100 gel filtration chromatography.
- The recombinant cystatin behaved as a thermal-stable protein and exhibited papain-like protease inhibition activity comparable to the natural chicken cystatin.

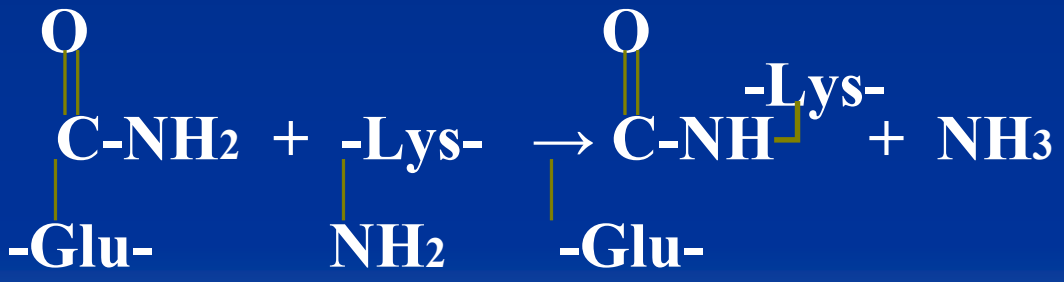
- **High level of the secreted form of recombinant chicken cystatin can be expressed in *Pichia pastoris* X-33 by chromosomal integration of multiple copies of an expression cassette containing chicken cystatin under the control of glyceraldehydes -3-phosphate dehydrogenase (GAP) promoter.**

- The inhibition ability of the recombinant against papain-like proteinase was found to be corresponding to those of natural chicken cystatin.
- The recombinant cystatin substantially inhibited the proteolysis of myosin and gel softening, which consequently improved the gel properties of mackerel surimi.

Production of MTGase and Its Application

Transglutaminase (TGase) widely distributes in animal tissues or organs, plants and microorganisms.

TGase can catalyze the formation of ε-(γ-glutamyl)lysine crosslinks.



ISSUE

- Limited source and complicated separation procedures increase the cost for obtaining this enzyme, which consequently make it not possible to apply in food processing.
- Mass production of this enzyme was probably the most essential issue.

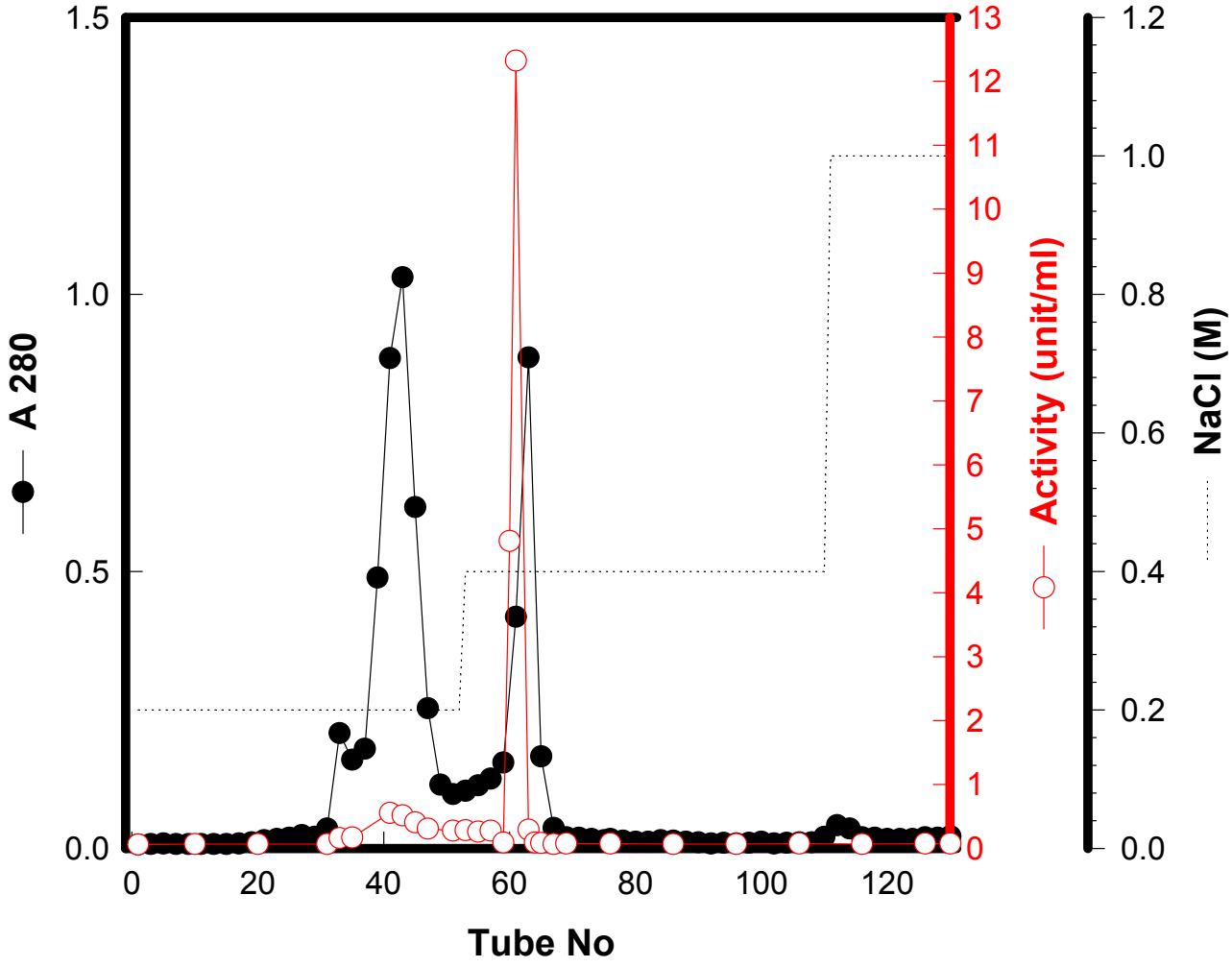
Production of microbial transglutaminase

- Motoki et al., 1989; Ando et al., 1989; Zeng et al., 2001: found the MTGase in cultures of *Streptoverticillium* sp. and *Streptomyces* sp.
- Our lab (Tsai et al., 1996a, b): found in culture of *Streptoverticillium ladakanum*

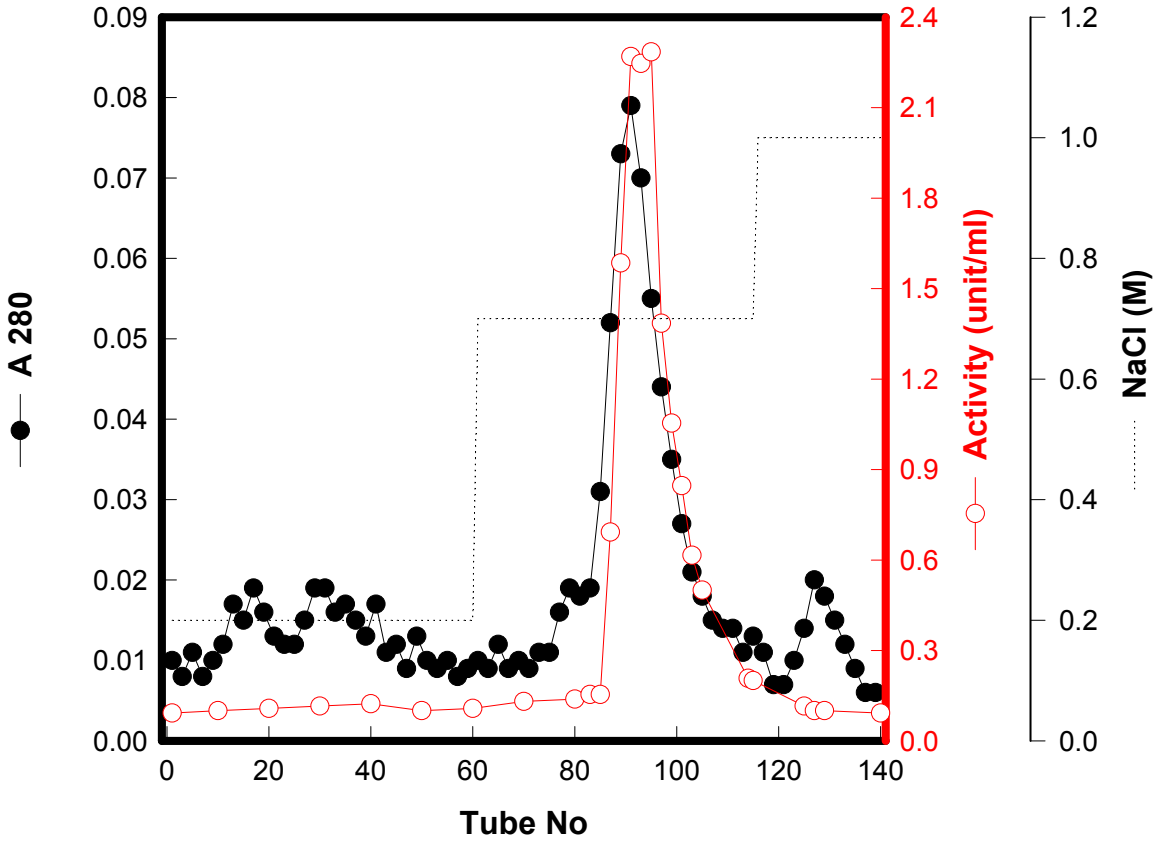
A modified process for purifying MTGase was developed in our lab (Ho et al. 2000).

- After the broth was centrifuged and filtered, the enzyme was purified directly with a rapid and simple stepwise chromatography method with CM Sepharose CL-6B and Blue Sepharose Fast Flow.
- This method is simple, rapid and has a MTGase recovery of 81%.
- Since MTGase is excreted into the culture medium, cell disruption is not necessary. Its purification thus proves to be rather easy. Consequently, its commercialization has been accelerated.

Chromatography of the crude MTGase from *Streptoverticillium ladakanum* on CM Sepharose CL-6B using stepwise elution method column: 2.6 x 40; V_o: 92 ml; washed with 50 mM Na-acetate buffer (pH 5.0, buffer C); eluted by 0.2 M and 0.4 M NaCl in buffer C; collection size: 4 ml/tube (1~110 tube), 10 ml/tube (111~130 tube); flow rate: 1.0 ml/min


A 280
Activity (unit/ml)
NaCl (M)
Tube No
1.5
1.0
0.5
0.0
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120

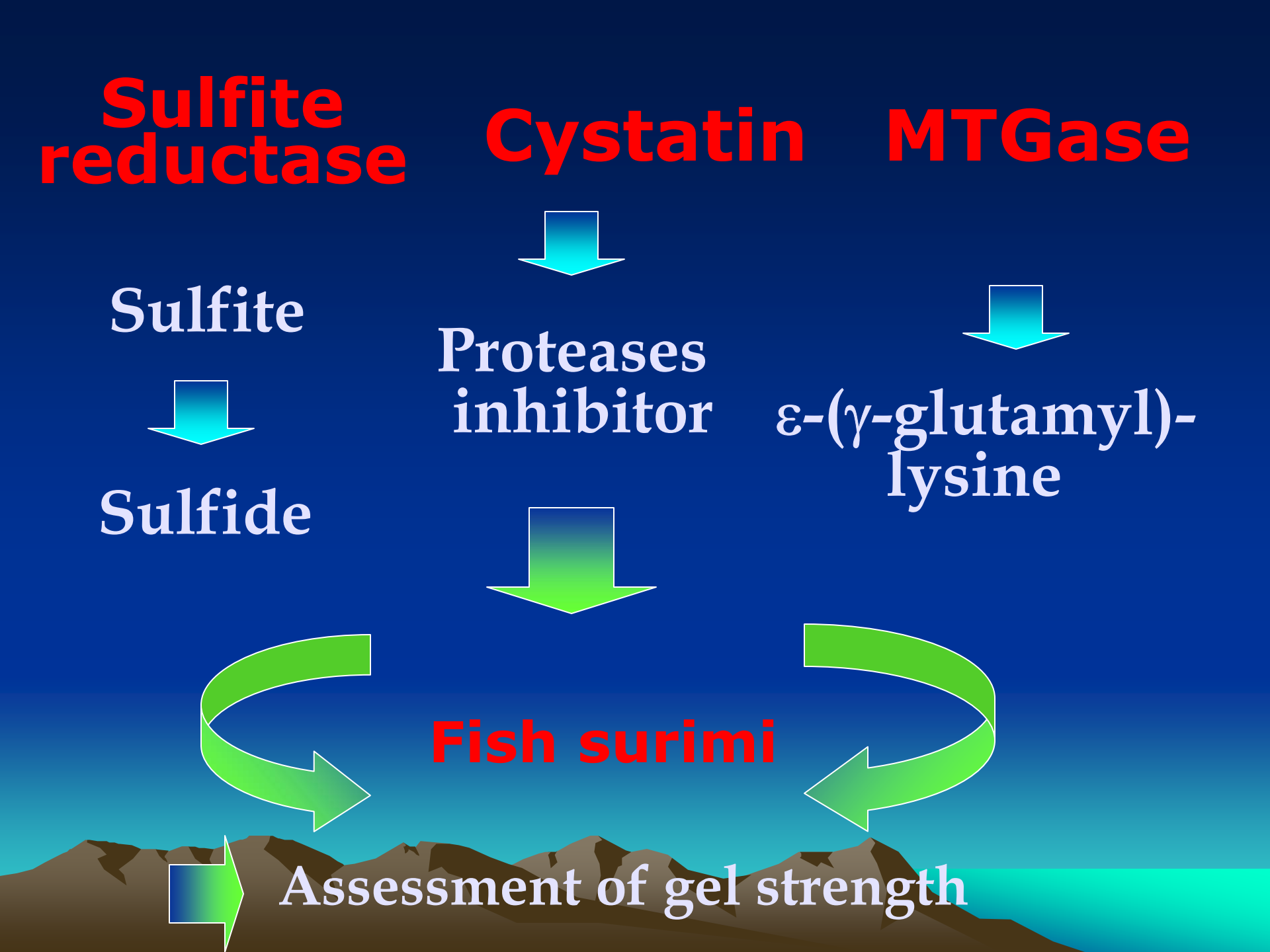
Chromatography of MTGase from *Streptoverticillium ladakanum* on Blue Sepharose Fast Flow using stepwise elution method column : 1.6 x 40; V_o: 22 ml; washed with 50 mM phosphate buffer (pH 7.0, buffer D); eluted by 0.2 M and 0.7 M NaCl in buffer D; collection size: 4 ml/tube (1-115 tube), 10 ml/tube (116-140 tube); flow rate : 1.0 ml/min.


A 280
Activity (unit/ml)
NaCl (M)
Tube No
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
2.4
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
20
40
60
80
100
120
140

Characteristics of MTGase

- The MW of TGase from microbial sources (30 to 45 kDa) is much lower than those from animals (77 to 90 kDa).
- The optimal temperature and pH of MTGase were 35-50^{o}C and 7.5-9.0.
- MTGase is calcium independent. Such a property is very useful in the modification of functional properties of food proteins, because many food proteins, such as milk caseins, soybean globulins and myosins, are susceptible to Ca^{2+}.

Application of Microbial Transglutaminase, Recombinant Cystatin and NADPH-sulfite Reductase on the Protein Gel Processing


Sulfite reductase
Cystatin
MTGase
Sulfite
Sulfide
Proteases inhibitor
ε-(γ-glutamyl)-lysine
Fish surimi
Assessment of gel strength

Preparation of cystatin, NADPH-sulfite reductase and MTGase

***Pichia* X-33**

(cystatin)

S. ladakanum
(MTGase)

S. cerevisiae
sulfite
(reductase)

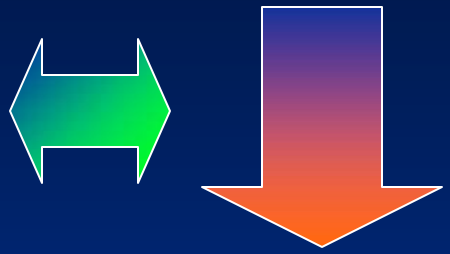
30°C for 3 days

28°C for 4 days

24°C for 60 hrs

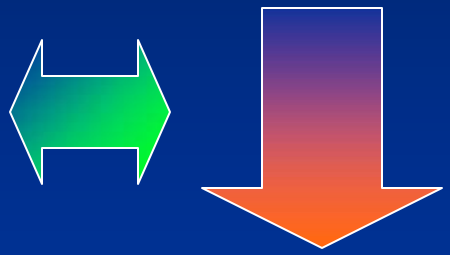
Mackerel surimi

with cystatin



Addition of 2.5 % NaCl

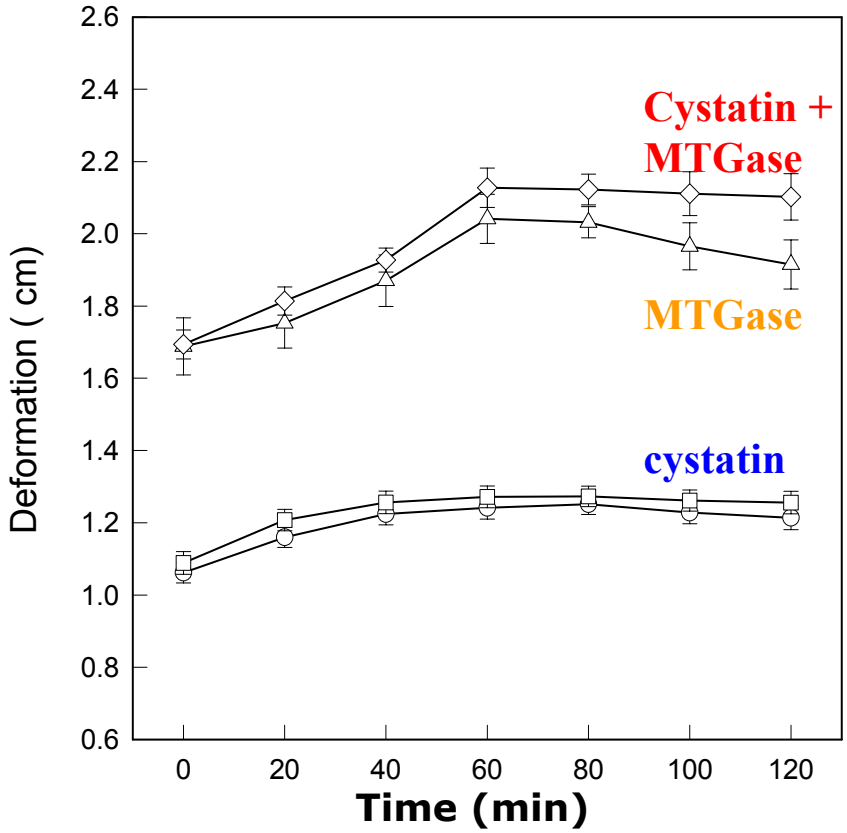
with MTGase



Setting

(30, 45°C for 0~2 hr)

Heated (90°C for 30 min)



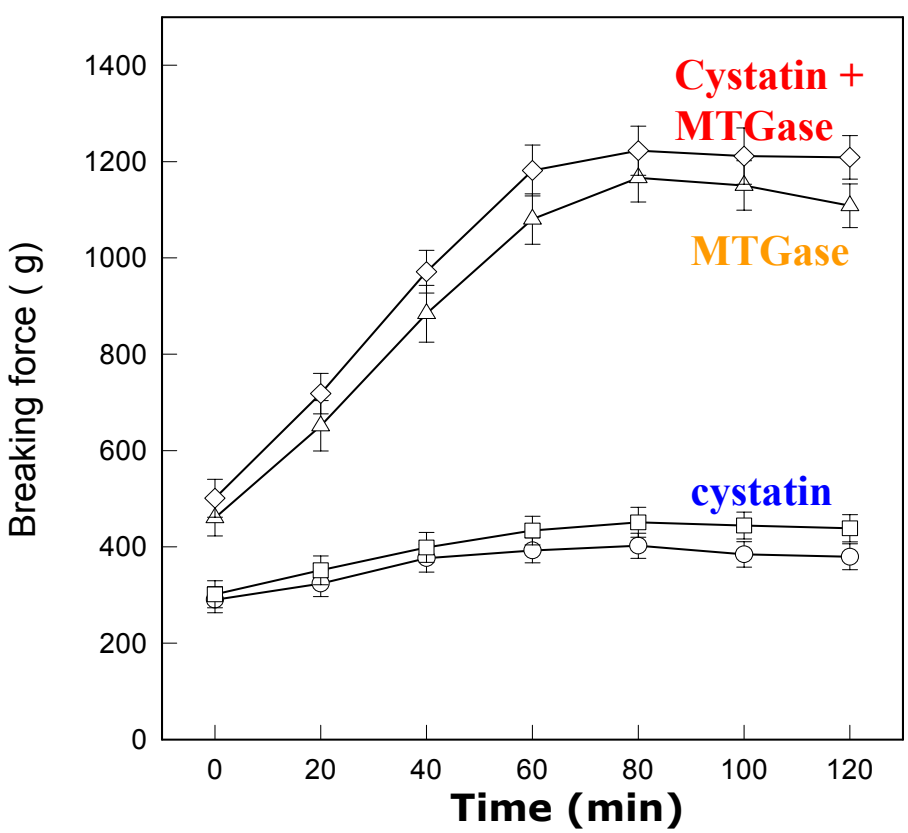


Figure 3. Changes in the breaking force and deformation of mackerel surimi with/without addition of MTGase and recombinant cystatin at 30°C. ○: Gels without additives; □: Gels with 0.04 units of recombinant cystatin/g; △: Gels with 0.5 units of MTGase/g; ◇: Gels with 0.04 units of recombinant cystatin/g and 0.5 units of MTGase/g.

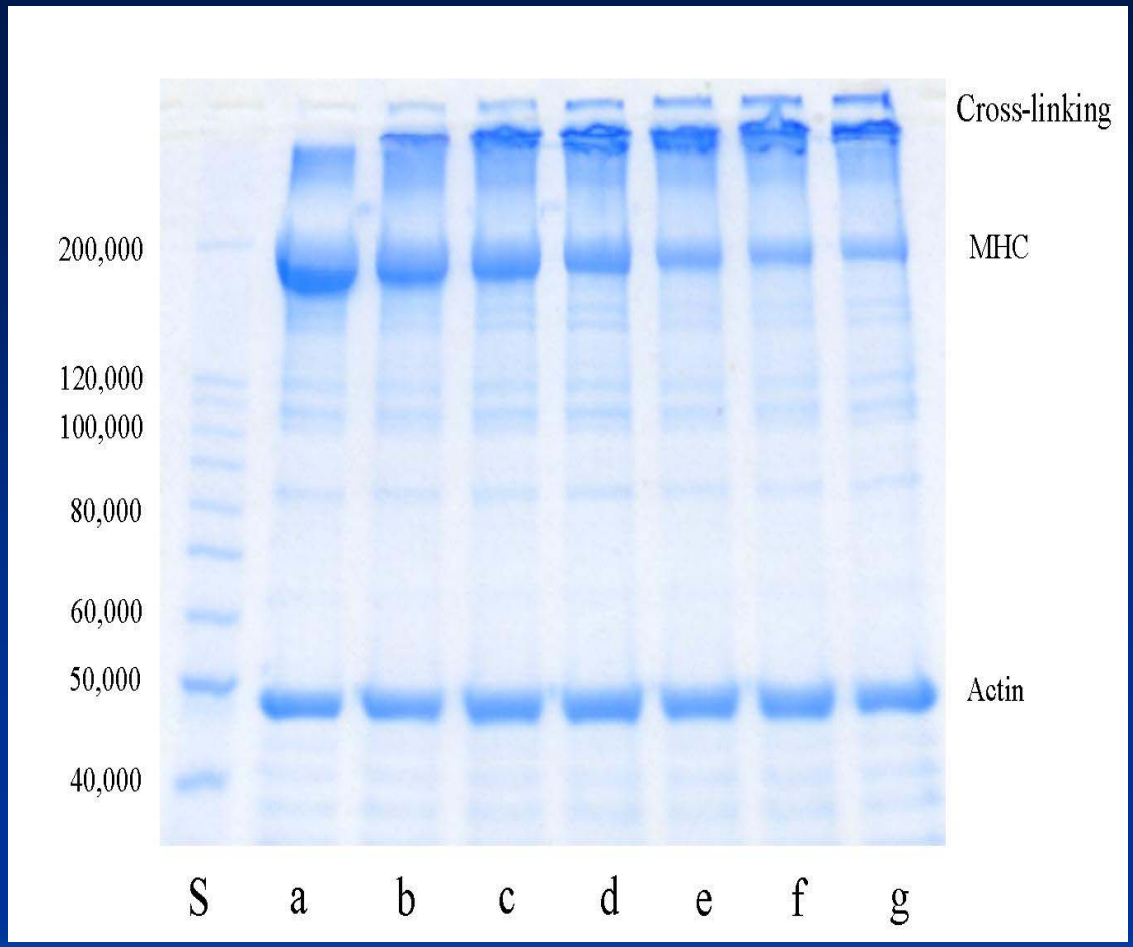


Figure 5. Change in SDS-PAGE profiles of mackerel AM with various amounts of MTGase incubated at 30°C for 30 min. (S: protein marker; a: 0 unit; b: 0.1 units; c: 0.2 units; d: 0.3 units; e: 0.4 units; f: 0.5 units; g: 0.6 units of MTGase/g AM)

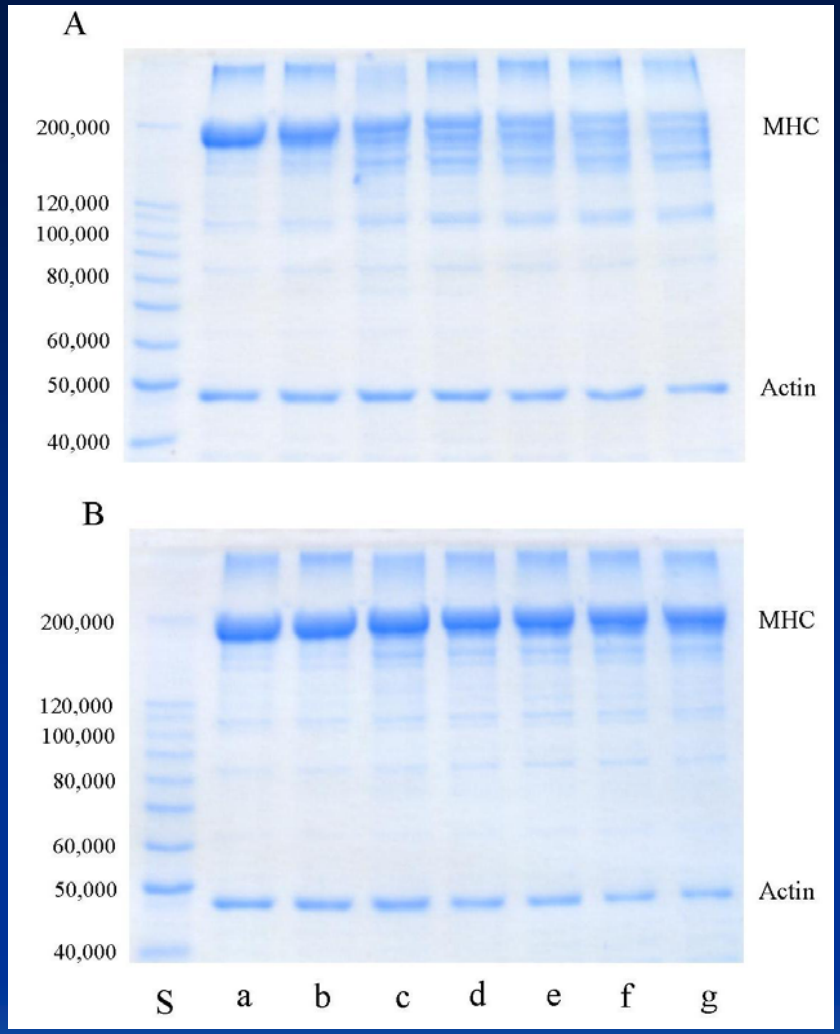


Figure 8. Change in SDS-PAGE profiles of mackerel AM with/without recombinant cystatin incubated at 45°C for various time periods. A: without recombinant cystatin; B: with 0.04 units of recombinant cystatin/g. (S: protein marker; a: 0 min; b: 20 min; c: 40 min; d: 60 min; e: 80 min; f: 100 min g: 120 min.)

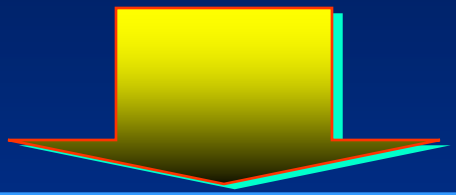
Conclusions:

- MTGase could catalyze the MHC cross-linking and increase the gel-forming ability of mackerel surimi.
- The texture softening caused by the endogenous protease could be substantially inhibited by the addition of recombinant cystatin.
- The combination use of MTGase and
- recombinant cystatin was considered to be more effective in improving the gel forming ability of mackerel surimi.

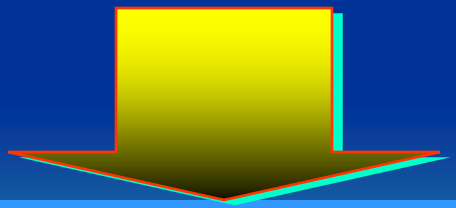
Summary of the application of sulfite reductase, recombinant cystatin and MTGase in surimi and food binders processings


Denatured Fish Muscle
Deboning & Leaching
Grinding

Addition of Reducing Agents or Sulfite Reductase



Add Recombinant Cystatin after adjusting the pH



Processed into Surimi

PROCESS FOR MAKING SURIMI-BASED PRODUCTS

Thanks for your attention