# Effects of Polymannosylation of Recombinant Cystatin C in Yeast on Its Stability and Activity

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The effects of glycosylation on stability and activity of recombinant cystatin were investigated using two yeast expression systems. A great difference in the length of polymannosyl chains in addition to 2 mol of *N*-acetylglucosamine was observed in mouse cystatin C produced by *Saccharomyces cerevisiae* and *Pichia pastoris* transformants. Polymannosylated cystatin with degree of polymerization (DP) of 310 (Cyst310) was predominantly produced by *S. cerevisiae* as a heterogeneous glycoprotein. In contrast, the DP of cystatin by *P. pastoris* was 90 (Cyst90). These yeast transformants also produced a small amount of oligomannosylated cystatin with DP of 13 (Cyst13) as well as unglycosylated protein. Susceptibility of Cyst310 and Cyst90 to α-chymotrypsin dramatically decreased to <10%, while that of Cyst13 was 77% of the susceptibility of unglycosylated cystatin. Polymannosylation improved the heat stability of cystatin to an extent that no coagulation was observed under the conditions, which coagulated unglycosylated protein. Papain-inhibiting activities of Cyst310, Cyst90, and Cyst13 were 18.5, 83.7, and 98.3% of that of unglycosylated cystatin, respectively. The retentions of inhibitory activity upon heating to 95 °C were 82.2 and 71.3% for Cyst310 and Cyst90, respectively, while those of Cyst13 and unglycosylated cystatin were below 10%. The polymannosylation of cystatin by *P. pastoris* is preferable to that by *S. cerevisiae* as the resulting protein is more stable and active in inhibiting papain.

**Keywords:** *Mouse cystatin C; Saccharomyces cerevisiae; Pichia pastoris; polymannosylation; protease susceptibility; heat stability*

## INTRODUCTION

Secretion of recombinant proteins in yeasts induces glycosylation at asparagine sites with the recognition sequences of Asn-X-Ser/Thr of polypeptides (Nakamura et al., 1993). The site-specific glycosylation of proteins using yeast expression systems is a new approach to enhance their molecular stability against heating and proteolysis. On the basis of papers reported in the literature with regard to safety in human consumption (Mendoza-Vega et al., 1994; Zuckerman, 1985), *Saccharomyces cerevisiae* has been accepted as a safe host organism to produce novel functional proteins. However, *S. cerevisiae* appears to be inappropriate to use for the production of biologically active proteins because of glycosylation with excessively long chains. Thus, the lytic activity of the polymannosyl lysozyme from *S. cerevisiae* greatly decreased to only 11% of the activity of the authentic lysozyme, when the insoluble cell wall of *Micrococcus lysodeikticus* was used as substrate (Nakamura et al., 1993).

Recently, the methylotrophic yeast *Pichia pastoris* was developed as an efficient system for the production of recombinant proteins with high expression levels (Cregg et al., 1993). Invertase secreted from recombinant *P. pastoris* was a relatively homogeneous family of molecular mass 85 000−90 000, whereas the enzyme from *S. cerevisiae* was much larger and more variable at 100 000−140 000 (Tschopp et al., 1987). It was also reported that the largest glycan isolated from *P. pastoris* was shorter than that from *S. cerevisiae* (Grinna and Tschopp, 1989). Thus, there is an increasing interest among researchers in investigating the effects of glycosylation level on protein stability and activity using these two yeast expression systems.

Cystatin C, an inhibitor of thiol proteinases, is ubiquitous in mammalian tissues and body fluids (Abrahamson et al., 1986, 1990; Esnard et al., 1988; Solem et al., 1990). This proteinase inhibitor has well-defined molecular structure of a relatively small size, having a great potential for application as a food and pharmaceutical aid (Turk and Bode, 1991). Therefore, we intended to employ this protein as a template for biosynthesis of novel glycoproteins. Basically, cystatin C is a member of family II in the superfamily of the cysteine protease inhibitors along with cystatin S (Barrett et al., 1986). Although it is assumed that cystatins belonging to families I and II are not glycosylated (Grubb and Löfberg, 1982), there is a report that a glycosylated cystatin C has been isolated from rat seminal vesicles accompanied with unglycosylated protein (Esnard et al., 1988). The existence of glycosylated rat cystatin was confirmed by analysis of the primary structure of cystatin C mRNA isolated from rat brain, in which a possible N-linked glycosylation sequence, Asn-Leu-Thr, was discovered at sites 79−81 (Cole et al., 1989). Possible sites for N-linked glycosylation were also found in mouse cystatin C cDNA cloned from mouse embryo, beginning from site 79 (Solem et al., 1990).

Of particular concern has been the site of N-linked glycosylation in a target protein when expressed in a

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S0021-8561(98)00121-6 CCC: $15.00 
Published on Web 06/03/1998

yeast expression system (Olsen and Thomsen, 1991; Nakamura et al., 1993; Kato et al., 1994; Choi and Jiménez-Flores, 1996). Rabies virus glycoprotein contains three potential glycosylation sites, two of which appear to be glycosylated in virions (Shakin-Eshleman et al., 1992). Human interleukin 5 has two putative N-linked glycosylation sites, Asn28 and Asn71, but only the former is glycosylated in Chinese hamster ovary cell (Minamitake et al., 1990). Ovalbumin has also two potential N-glycosylation sites at positions 293 and 312, of which only the former is glycosylated in vivo (Sheares, 1988). Four mutant lysozymes (G49N, G69N, P70N, and M105T) were constructed to have a specific recognition site for N-glycosylation; however, only the G49N mutant lysozyme was secreted in the two types of glycosylated forms with an oligosaccharide chain and a polysaccharide chain (Nakamura et al., 1993). Thus, by selecting a mouse cystatin C gene, which possesses a potential site for N-linked glycosylation at Asn79, we could produce novel glycoproteins of cystatin C without specific selection of the glycosylation site.

This paper deals with the effects of polymannosylation on α-chymotrypsin hydrolysis and heat denaturation of recombinant cystatins as well as their proteinase-inhibiting activity.

## MATERIALS AND METHODS

**Materials.** *S. cerevisiae* BJ3501 (*MATa pep4::HIS3 pro1-Δ1 6R his3-Δ200 ura3−52 can1 gal2*) and *P. pastoris* GS115 (*his4*) were used as hosts in this study. *Escherichia coli* JM 109 [*recA endA1 gyrA96 thi hsdR17 supE44 relA1* Δ *(lac-proAB) mcrA F′ traΔ36 proA+ proB+ lacIq lacZΔM15*] was used for routine plasmid amplification. Complementary DNA of mouse cystatin C (Solem et al., 1990) was provided by Dr. D. Barnes, Oregon State University. The yeast expression plasmid pVT100-U containing the entire *ADH1* gene promoter (Vernet et al., 1987) was a gift from Dr. T. Vernet, National Research Council of Canada. T4DNA ligase, alkaline phosphatase, and restriction enzymes were purchased from Promega (Madison, WI). Q-Sepharose Fast Flow, Sepharose 4B, Sephacryl S-100 HR, and concanavalin A−Sepharose were from Pharmacia (Baied'Urfe, QC). Endo-*β*-*N*-acetylglucosaminidase was purchased from Boehringer Mannheim (Laval, QC). Papain (type III), α-*N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA) and tunicamycin were from Sigma (St. Louis, MO). All other chemicals were of analytical grade for biochemical use.

**Construction of Yeast Expression Plasmids, Transformation of Yeast, and Culture Condition.** For the construction of *S. cerevisiae* expression plasmid, a 483 bp *Xba*I fragment containing mouse cystatin C gene (Solem et al., 1990) was inserted into the multiple cloning site of the pVT100-U vector between the *ADH1* promoter and terminator. The expression vector was introduced into *S. cerevisiae* BJ3501 according to the lithium acetate procedure (Itoh et al., 1983). Ura+ transformants were screened by subculturing on modified Burkholder minimum medium (Toh-e et al., 1981) plates supplemented with histidine (20 μg/mL) at 30 °C. After cultivation, well-growing colonies were replica-cultivated in the yeast minimum medium on a small scale (5 mL). The subclones with the highest levels of cystatin activity were then propagated from single colonies. The overexpression colonies were directly subcultured on a large scale (1 L) in the yeast medium at 30 °C for 5 days. On the other hand, the construction of *P. pastoris* expression vector was performed according to the method described in the manual of the *Pichia* expression kit (Invitrogen, San Diego, CA).

To express the cystatin gene at a native N terminus (Ala), an *Xho*I site and the signal sequence of the *Kex*2 cleavage (Glu-Lys-Arg) were introduced upstream of the N-terminal amino acid of cystatin by polymerase chain reaction (PCR) using 5′-AGG CTC GAG AAA AGA GCG ACC CCA AAA CAA-3′ as primer. The resulting vector was integrated into the AOX genome of *P. pastoris* GS115 by spheroplast transformation after linearization with *Dra*I according to the protocol in the manual. Integration of cystatin C in the recombinant genomes of *P. pastoris* was confirmed by genomic PCR using two primers, 5′-GAC TGG TTC CAA TTG ACA AGC-3′ and 5′-GCA AAT GGC ATT CTG ACA TCC-3′, as described in the manual. Transformants were grown in 5 mL of the yeast minimum medium containing 0.4 mg/L biotin at 30 °C for 24 h and then subcultured in 1 L of fresh yeast medium containing 0.5% methanol as the sole carbon source.

**Purification.** A culture of the recombinant yeast was centrifuged at 10000*g* for 10 min, and the supernatant was concentrated using an ultrafiltration system with 3000 molecular weight cutoff (Pellicon cassette filter, PLBC 000 05, Millipore, Bedford, MA). The concentrated culture was applied to a Q-Sepharose Fast Flow column (1.3 × 10 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was washed with the same buffer until protein-free fractions were obtained, and then the bound protein was eluted with a linear gradient to 500 mM NaCl. Fractions exhibiting papain-inhibiting activity were combined and applied to a Sephacryl S-100 HR column (1.3 × 75 cm) after concentration by pressure ultrafiltration using a Diaflo YM2 membrane (Amicon, Danvers, MA). The gel filtration column was equilibrated and eluted with 20 mM Tris-HCl buffer (pH 7.5). Protein content in each fraction was determined by measuring absorbance at 280 nm, and carbohydrate content was measured from absorbance at 490 nm after color development using the phenol−sulfuric acid reaction (Dubois et al., 1956).

The unglycosylated cystatin was further purified using CM-papain−Sepharose 4B (1.3 × 5 cm) as described by Barrett (1981). Meanwhile, all fractions containing glycoproteins were combined and applied to a concanavalin A−Sepharose column (1.3 × 5 cm) previously washed with 10 bed volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl and then washed with the same buffer until the washing solution was free from proteins. The adsorbed glycoprotein was subsequently eluted with 100 mM methyl α-mannoside in the 20 mM Tris-HCl (pH 7.5) buffer containing 0.5 M NaCl and used for further experiments after dialysis against deionized water.

**Characterization.** Susceptibility of the glycosylated cystatin C to endo-*β*-*N*-acetylglucosaminidase (endo-H) was investigated according to the method of Tarentino and Maley (1974). A sample of 0.2 mg/mL was boiled in 50 mM sodium citrate buffer (pH 5.5) containing 1% SDS and 200 μg/mL phenylmethanesulfonyl fluoride (PMSF) for 5 min. After incubation with an equal volume of 0.02 unit of endo-H in the same buffer at 37 °C for 20 h, the boiled sample was analyzed by SDS−polyacrylamide gel electrophoresis (SDS−PAGE).

SDS−PAGE was carried out according to the method of Laemmli (1970) using 15% acrylamide separating gels and 5% stacking gels containing 1% SDS. Samples were heated at 100 °C for 5 min in Tris-glycine buffer (pH 8.8) containing 1% SDS and 1% 2-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 electrophoresis, the gel sheets were stained for protein and carbohydrate with 0.025% Coomassie Brilliant Blue R-250 and 0.5% periodic acid−Fuchsin solution (Zachariou et al., 1969), respectively.

The N-terminal amino acid sequence was determined using an Applied Biosystems (Foster, CA) gas-phase sequencer equipped with an on-line amino acid phenylthiohydantoin analyzer after automated Edman degradation. The total sugar content of the glycosylated cystatins was estimated using the phenol−sulfuric acid reaction (Dubois et al., 1956) employing mannose as a standard. HPLC analysis was used for identifying the hexose liberated from glycosylated cystatins by hydrolysis with 2 N HCl at 100 °C for 3 h in a sealed glass ampule. The hydrolysates were dried, dissolved in water, and chromatographed on an Asahipak NH2P-50 column (Asahi Chemical, Tokyo) in 75% acetonitrile using the Hitachi (Tokyo) HPLC system equipped with an RI detector.

**Papain-Inhibiting Activity**. Papain inhibition assay was carried out to determine the activity of recombinant cystatins. The proteolytic activity of papain was measured using BAPNA as substrate according to the method of Arnon (1970). Mercuripapain (0.5 mg/mL) and inhibitor solution, 0.1 mL each, were mixed and added to 1.0 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 5 mM cysteine. After incubation for 25 min at 37 °C, the reaction was stopped by adding 0.2 mL of 30% acetic acid. The absorbance of the reaction product was measured at 410 nm. The enzyme activity of papain was calculated using the molar extinction of *p*-nitroaniline at 410 nm ($E_{1\%} = 8800$). One unit of enzyme activity was expressed as the activity that hydrolyzed 1 $\mu$mol of BAPNA/min (Arnon, 1970).

**Protein Stability.** The stability of the recombinant cystatins was measured using α-chymotrypsin hydrolysis and thermal denaturation. Susceptibility to α-chymotrypsin was assessed according to the procedure reported by Akita and Nakai (1990). A 0.1% protein solution in 20 mM Tris-HCl buffer (pH 7.5) was mixed with 0.1 volume of 0.1% α-chymotrypsin at 37 °C for 0, 1, 2, 3, 5, 10, 20, and 40 min. After incubation, aliquots of the digests were taken, and the content of free amino groups in the heated samples was determined by the trinitrobenzenesulfonate method (Haynes et al., 1967). The release of small peptides and amino acids increased almost linearly with time during the first 5 min. The digestion velocity was calculated as the amount of amino acids or peptides produced per minute from the initial slope of hydrolysis.

Apparent heat stability was estimated by measuring the turbidity developed upon heating as previously described (Nakamura et al., 1993). The recombinant cystatins were heated to 95 °C at a rate of 1 °C/min from 30 °C in 50 mM sodium phosphate buffer (pH 7.5) at a protein concentration of 1 mg/mL. At preset temperatures, each heated sample was transferred into a cuvette and the turbidity measured at 500 nm. The residual papain-inhibiting activity of the heated samples was also measured as described above under Papain-Inhibiting Activity. This procedure was repeated in triplicate.

## RESULTS AND DISCUSSION

**Expression of Mouse Cystatin C in Yeast.** Recombinant cystatins secreted from transformants of *S. cerevisiae* and *P. pastoris* were collected by ion exchange chromatography using a Q-Sepharose Fast Flow column. The active inhibitory fractions were eluted from the column with 200 mM NaCl in 20 mM Tris-HCl buffer (pH 7.5) as shown in Figure 1. All active fractions were pooled and further purified into unglycosylated, oligoglycosylated, and polyglycosylated forms using CM-papain−Sepharose 4B followed by concanavalin A−Sepharose columns after gel filtration through a Sephacryl S-100 HR column (data not shown).

Figure 2 shows SDS−PAGE patterns of the active fractions indicated in Figure 1. A great difference in the molecular mass was observed between the recombinant cystatins secreted from *S. cerevisiae* and *P. pastoris* transformants as reported by Cregg et al. (1993). Long-chain glycosylated cystatin was predominantly expressed in *S. cerevisiae*. In contrast, the degree of glycan polymerization in glycosylated cystatin from *P. pastoris* was appreciably less than that from *S. cerevisiae*. Both yeast strains produced small amounts of oligoglycosylated cystatins as well as unglycosylated protein. Yields of recombinant cystatins were in total 1.3 and 110 mg/L in *S. cerevisiae* and *P. pastoris*, respectively.

The glycosylation type of oligo- and polyglycosylated cystatins was determined by treatment with endo-$\beta$-*N*-acetylglucosaminidase, which generally catalyzed deglycosylation, thereby yielding high-mannose type sugar

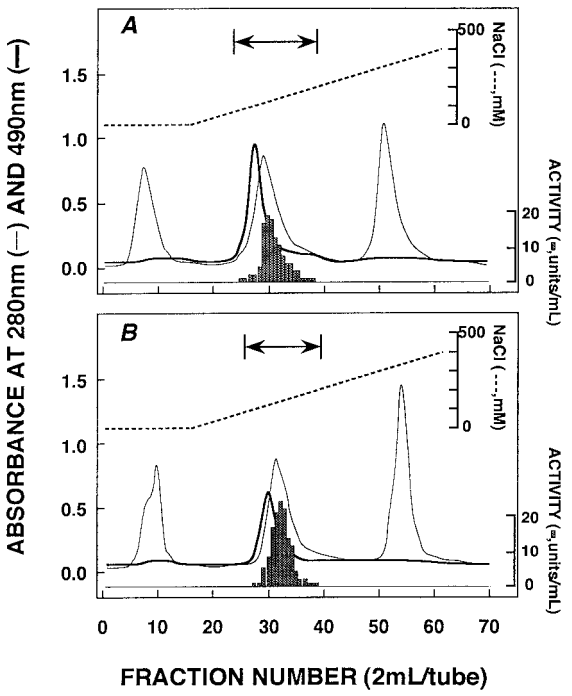


**Figure 1.** Isolation of recombinant cystatins secreted from *S. cerevisiae* (A) and *P. pastoris* (B) using a Q-Sepharose Fast Flow column. The thin and thick lines indicate the absorbances of protein and carbohydrate, respectively. The shadow bars indicate the inhibitory activity to papain. The fractions indicated by horizontal arrows were collected and used for further experiments.

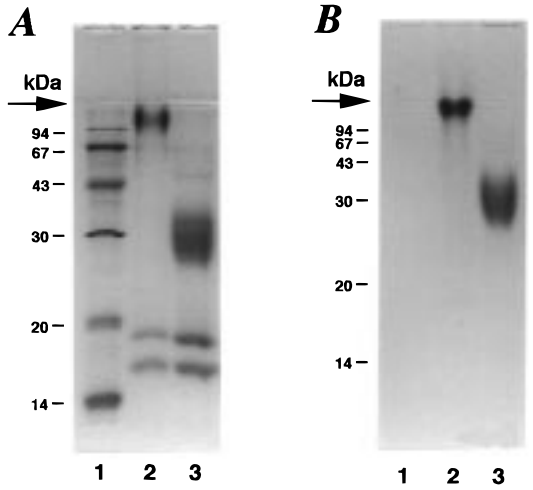


**Figure 2.** SDS−PAGE patterns of recombinant cystatins secreted from *S. cerevisiae* and *P. pastoris*. Electrophoresis was carried out at a constant current of 10 mA for 5 h in Tris/glycine buffer (pH 8.8) containing 0.1% SDS. The gel sheets were stained for proteins and carbohydrates with Coomassie Brilliant Blue (A) and periodic acid−Fuchsin (B), respectively. Arrows indicate the position of boundary between the stacking (upper) and separating (lower) gels.

chains. As shown in Figure 3, all glycosylated cystatins were completely digested by the glycosidase. To verify the type of glycosylation, a further test using tunicamycin, which effectively prevented the N-linked mannose incorporation into protein (Elbein, 1987), was conducted. There was no glycosylation in the protein recovered from the yeast culture supplemented with 1 mg of tunicamycin/L (data not shown). Thus, it was confirmed that the mouse cystatin C gene carrying one potential site for N-linked glycosylation at Asn79 was

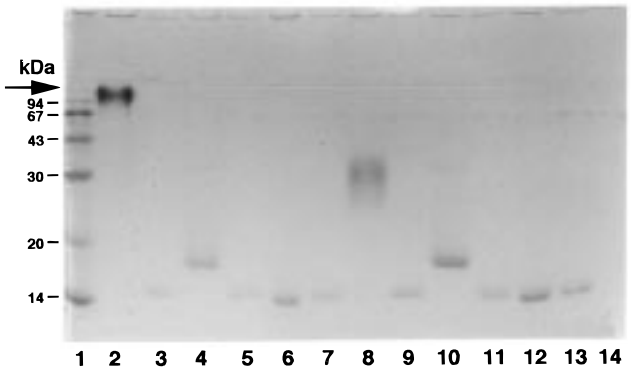


**Figure 3.** Endo-H treatment of oligo- and polyglycosylated cystatins purified by affinity chromatography. The glycosylated proteins were boiled with 50 mM sodium citrate buffer (pH 5.5) containing 1% SDS and 200 μg/mL of phenylmethanesulfonyl fluoride for 5 min and then incubated with 0.02 unit of endo-H at 37 °C for 20 h. Endo-H digests were subjected to SDS−PAGE. The gel sheet was stained with Coomassie Brilliant Blue. The arrow indicates the position of the boundary between the stacking (upper) and separating (lower) gels. Lane 1, molecular weight markers (same as in Figure 2); lane 2, polyglycosylated cystatin from *S. cerevisiae*; lane 3, endo-H digest of polyglycosylated cystatin from *S. cerevisiae*; lane 4, oligoglycosylated cystatin from *S. cerevisiae*; lane 5, endo-H digest of oligoglycosylated cystatin from *S. cerevisiae*; lane 6, unglycosylated cystatin from *S. cerevisiae*; lane 7, endo-H digest of unglycosylated cystatin from *S. cerevisiae*; lane 8, polyglycosylated cystatin from *P. pastoris*; lane 9, endo-H digest of polyglycosylated cystatin from *P. pastoris*; lane 10, oligoglycosylated cystatin from *P. pastoris*; lane 11, endo-H digest of oligoglycosylated cystatin from *P. pastoris*; lane 12, unglycosylated cystatin from *P. pastoris*; lane 13, endo-H digest of unglycosylated cystatin from *P. pastoris*; lane 14, endo-H.

**Table 1. Carbohydrate Composition of Oligo- and Polymannosyl Cystatins Expressed in *S. cerevisiae* and *P. pastoris***

| | | contents, mol/mol of cystatin | |
|---|---|---|---|
| glycosylation type | host yeast | *N*-acetylglucosamine[a] | mannose[b] |
| oligomannosylation | *S. cerevisiae* | 2 | 13 |
| | *P. pastoris* | 2 | 13 |
| polymannosylation | *S. cerevisiae* | 2 | 310 |
| | *P. pastoris* | 2 | 90 |

[a] Determined with an amino acid analyzer using the hydrolysates with 3 N HCl at 100 °C for 4 h. [b] Determined by HPLC analysis using an NH2P-50 column and by the phenol−sulfuric acid method. Each value is the average of three replications.

expressed as two types of polyglycosyl cystatins, with N-linked polysaccharide chains in addition to an oligoglycosyl form in *S. cerevisiae* and *P. pastoris*.

Chemical composition analysis revealed that the polysaccharide chain of the polyglycosyl cystatin secreted from *S. cerevisiae* consisted of 310 mol of mannose and 2 mol of of *N*-acetylglucosamine, while that of *P. pastoris* consisted of 90 and 2 mol, respectively (Table 1). This result indicates that the polyglycosyl cystatins from *S. cerevisiae* and *P. pastoris* carry 310Man-2GlcNAc-linked (Cyst310) and 90Man-2GlcNAc-linked forms (Cyst90), respectively. Meanwhile, the oligosaccharide composition of 13Man-2GlcNAc was determined in the oligoglycosylated cystatins from both *S. cerevisiae* and *P. pastoris* (Cyst13). This result corresponds to an increase in apparent molecular mass of ~3 kDa in SDS−PAGE (Figure 3). Oligomannosylated cystatin from *P. pastoris* was used as Cyst13 for further investigation.

These recombinant cystatins were analyzed for their amino acid sequences from the N terminus using an automatic gas-phase protein sequencer. All N-terminal sequences of Cyst310, Cyst90, and Cyst13 were $NH_2$-ATPKQGPRML, identical to the authentic protein sequence (Solem et al., 1990). This result indicates that these mature proteins are correctly processed during the expression in the host cells.

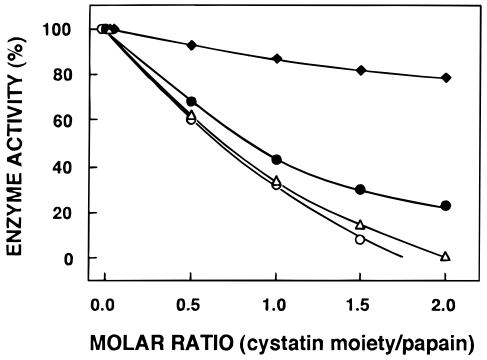


**Figure 4.** Inhibition of the papain activity by the recombinant cystatins. 0.1 mL Papain (0.1 mL; 0.5 mg/mL) and inhibitor (0.1 mL) solution were preincubated in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM EDTA and 5 mM cysteine. After incubation for 25 min at 37 °C, the remaining activity of papain was determined using BAPNA as substrate. ○, unglycosylated cystatin; △, oligomannosylated cystatin from *P. pastoris* (Cyst13); ●, polymannosyl cystatin from *P. pastoris* (Cyst90); ◆, polymannosyl cystatin from *S. cerevisiae* (Cyst310).

**Effects of Polymannosylation on the Papain-Inhibiting Activity of Cystatins.** The effect of oligo- and polymannosylation of cystatins on papain-inhibiting activity was investigated using BAPNA as substrate. Stoichiometric titration curves of papain with recombinant cystatins are shown in Figure 4. Biological activity of the mouse cystatin C was not appreciably deteriorated by the oligomannosylation. The inhibition profile of Cyst13 was almost the same as that of unglycosylated cystatin. Polymannosyl cystatin from *P. pastoris* (Cyst90) was considerably more effective than Cyst310 from *S. cerevisiae* in inhibiting the papain activity. The inhibitory activity was highly conserved in the moderately polymannosylated cystatin from *P. pastoris* (Cyst90), whereas it was significantly decreased in the fully polymannosylated cystatin from *S. cerevisiae* (Cyst310). This result indicates that the long polymannosyl chain of protein from *S. cerevisiae* may interfere with effective interaction between papain and the recombinant cystatin.

A relative activity of the recombinant cystatins was calculated on the basis of the residual activity of papain when 1 mol of the proteinase was incubated with 1 mol of cystatin moiety. The papain-inhibiting activities of Cyst13, Cyst90, and Cyst310 were 98.3, 83.7, and 18.5% of that of unglycosylated cystatin, respectively. The long-chain glycosylation in *S. cerevisiae* may be unsuitable for producing biologically active proteins.

**Improved Stability of Cystatin by Polymannosylation.** The stability of the recombinant cystatins was analyzed by proteolysis and heat denaturation. First, the susceptibility of unglycosylated cystatin to α-chymotrypsin activity was compared with the three glycosylated derivatives of recombinant cystatins, which differed in polymannosyl chain length. As shown in Table 2, a low initial rate of hydrolysis was obtained for Cyst310 and Cyst90. The susceptibility to α-chymotrypsin of Cyst310 and Cyst90 dramatically decreased to <10% of that of unglycosylated cystatin, while that of Cyst13 was 77%. On the basis of the

**Table 2. Protease Susceptibility of Oligo- and Polymannosyl Cystatins against α-Chymotrypsin**

| sample protein | rel initial rate of hydrolysis[a] |
|---|---|
| unglycosylated cystatin | 1.00 |
| Cyst13 (oligomannosylated cystatin) | 0.77 ± 0.09 |
| Cyst90 (polymannosylated cystatin from *P. pastoris*) | 0.09 ± 0.02 |
| Cyst310 (polymannosylated cystatin from *S. cerevisiae*) | 0.09 ± 0.01 |

[a] Sample protein (0.1%) was exposed with $^1/_{10}$ volume of 0.1% α-chymotrypsin (see Materials and Methods for experimental details). Values are reported relative to the nonglycosyl cystatin, means ± SD.

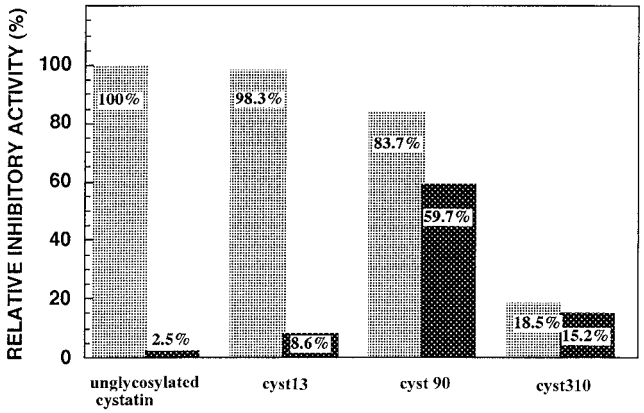


**Figure 5.** Relative inhibitory activity of the recombinant cystatins to papain before and after heating. Heating process was performed for a 0.1% protein solution in 50 mM sodium phosphate buffer (pH 7.5) by heating to 95 °C at a rate of 1 °C/min from 30 °C. Lightly shaded bar, unheated sample; heavily shaded bar, heated sample. The data were the averages of triplicate determinations.

enzymatic removal of bulky oligosaccharides, there was circumstantial evidence that carbohydrate moieties contributed to protection of the polypeptide portions of glycoproteins from proteolysis (Olden et al., 1985; Gu et al., 1989). In this study, we found that the polymannosylation of protein alone reduced the proteinase susceptibility of glycoproteins, with both Cyst310 and Cyst90 only slightly hydrolyzed with α-chymotrypsin. It is possible that polymannosyl chains can disturb the physical accessibility of α-chymotrypsin to the proteolytic cleavage sites in the polymannosyl cystatins.

The heat stability of the recombinant cystatins was assessed by monitoring their thermal denaturation when a 0.1% protein solution was heated to 95 °C. This heating assay revealed that polymannosylation improved the heat stability of cystatin to an extent that no coagulation was observed. Under the same conditions, unglycosylated protein had coagulated. Although it is well-known that cystatin C is highly resistant to heating due to the tightly packed conformation, >10% reduction of soluble protein was observed at 75 °C in the unglycosylated sample, indicating a protein aggregation. Upon heating, the protein molecules are partially unfolded, resulting in aggregation due to the heat-induced disruption of a delicate balance of various noncovalent interactions. This process is reversible in the glycosylated cystatins because of the inhibition of aggregation by polymannosyl chains through interaction of unfolded proteins, which is irreversible in the case of unglycosylated protein (Nakamura et al., 1993).

Figure 5 shows the relative inhibitory activity of the recombinant cystatin C before and after heating to 95 °C at a rate of 1 °C/min. After heating to 95 °C, a polymannosyl cystatin, Cyst90, showed ~60% of the inhibition made by native unglycosylated cystatin C, which was the highest activity among all heated samples. This implies the retention of 71.3% of the inhibitory activity upon heating. On the other hand, the relative activity of Cyst310 decreased only slightly, from 18.5 to 15.2%, upon heating, implying 82.2% activity retention. In contrast, the protection of cystatin C from heat instabilization by oligomannosylation (Cyst13) was slight, with an activity retention of only 6.1% (Figure 5). Overall, the chain length of the glycan added is the most important factor in the maintenance of the biological activity of the proteinase inhibitor. The moderate polymannosylation in *P. pastoris* may be preferable for producing a stable, biologically active inhibitor to thiol proteinases. Although cystatin is a potent inhibitor of sulfhydryl proteases, it is vulnerable to the attack of other endogenous proteases. Cystatin C was inactivated by hydrolysis of hydrophobic sites with cathepsin D, an aspartyl proteinase (Lenarčič et al., 1991). The improved stability seen here with polyglycosylated cystatins could be beneficial in avoiding the risk of degradation during applications of this protease inhibitor.

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Received for review February 10, 1998. Revised manuscript received April 20, 1998. Accepted April 22, 1998.

JF9801217