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Effect of Glycosylation Modification (N-Q-^{108}I → N-Q-^{108}T) on the Freezing Stability of Recombinant Chicken Cystatin Overexpressed in *Pichia pastoris* X-33

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The cDNAs encoding chicken cystatin and its N-glycosylation-modified mutant (Asn_{106}−Ile_{108}→Asn_{106}−Thr_{108}) were cloned into the pGAPZαC expression vector, using the GAP as promoter and Zeocin as resistant agent, and transformed into *Pichia pastoris* X-33 expression host. The effect of N-glycosylation on the stability of recombinant chicken cystatin was investigated. A large quantity of recombinant chicken cystatin and the Asn_{106}-glycosylated cystatins were expressed and secreted into broth using α-factor preprosequence. The K_i of the recombinant chicken cystatin (0.08 nM) was similar to that of wild-type chicken cystatin (0.05 nM). They acted as a competitive inhibition reaction against papain. According to the K_i, the inhibition ability of Asn_{106}-glycosylated mutant cystatin (K_i = 9.5 nM) was weaker than that of the wild-type one. However, N-glycosylation at Asn_{106} substantially enhanced the freezing stability of recombinant chicken cystatin overexpressed in *P. pastoris*.

KEYWORDS: ***P. pastoris*; glycosylation modification; recombinant cystatin; overexpression**

INTRODUCTION

The cystatin superfamily includes a number of cysteine proteinase inhibitors that are widely distributed in the tissues and body fluids of mammalians (*1*). Chicken cystatin, a well-studied cysteine proteinase inhibitor, is a small nonglycosylated protein (116 amino acids) with two disulfide bonds (*1*−*5*). This inhibitor has been crystallized and subjected to preliminary X-ray crystallographic studies (*6*, *7*). It was found that chicken cystatin is stable to heat (*4*) but unstable to freezing or freeze-drying (*2*, *8*). This is a reverse, tight-binding inhibitor of papain-like enzymes and is considered to contribute to physiological control of the proteinases (*9*−*12*). However, the application of cystatin on inhibition of autolysis or endogenous proteolysis was greatly restricted by its lower freezing tolerance. To expand the use of this cystatin to frozen products, the cystatin moiety may need to be modified to increase the flexibility and resistance to freezing process. Therefore, we intended to employ this protein as a template for the biosynthesis of novel glycoproteins and investigate the effect of glycosylation on protein expression and freezing stability.

The site-specific glycosylation of proteins using yeast expression systems is a new approach to enhance the molecular stability of recombinant protein (*13*−*15*). *Pichia pastoris*, the methylotrophic yeast, is an efficient system for the production of recombinant proteins with a high expression level (*16*, *17*). The glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter gene has been characterized and can express recombinant proteins to high levels in *P. pastoris* (*18*). To examine the effect of N-glycosylation on the freezing stability of recombinant chicken cystatin, the cDNAs coding chicken cystatin and its N-glycosylated mutant (Asn_{106}−Ile_{108} → Asn_{106}−Thr_{108}) were cloned into the pGAPZαC expression vector, using the GAP as promoter and Zeocin as resistant agent, and transformed into *P. pastoris* X-33 expression host. The papain inhibition properties of the recombinant chicken cystatin and Asn_{106}-glycosylated mutant cystatin were investigated.

MATERIALS AND METHODS

Screening and Amplification of Cystatin cDNA from Chicken Lung mRNA. Total RNA from chicken lung was extracted using a Trizol RNA extraction kit (Gibco BRL, Gaithersburg, MD). The single-strain cDNA, produced from RT-PCR, was used as a template. On the basis of the open reading frame from 128 to 478 of chicken cystatin, the oligonucleotides with 5′-CTCGAGAAAAGAGAGGCTGAAGCT-AGCGAGGACCGCTCCCGGCTCCTGGG and 5′-TCTAGATTACTG-GCACTTGCTTTCCAGCAGTTT were used as primers for the PCR reactions. Another antisense primer, TCTAGATTACTGGCACT-TGCTTTCCAGCAGTTTA**G**TTTGG, in which the ^{106}Asn-Gln-^{108}Ile was replaced by Asn-Gln-Thr (boldface) to create a glycosylation sequence, was used to substitute the 3′-primer in the PCR reaction. Restriction sites at the 5′ end of the primers for *Xho*I and *Xba*I (underscored) were incorporated to facilitate subcloning of the product.

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Amplification was performed using proofreading polymerase (Gibco BRL) by Polymerase Chain Reaction for 35 cycles with 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 50 s of extension at 68 °C in a DNA thermal cycler (GeneAmp PCR system 2400; Perkin-Elmer, Norwalk, CT).

Construction of Cystatin Expression pGAPZαC Plasmid. The standard techniques of molecular cloning were performed mainly according to the method of Sambrook et al. (*19*). The PCR product was cloned according to the method of Sambrook et al. (*19*) into pGEMT Easy vector (Promega, Madison, WI) and then transformed into *Escherichia coli* Top 10 F′. After blue/white selection and midipreparation, the plasmid was digested with *Xho*I and *Xba*I and then ligated into pGAPZαC vector (Novagen Inc., Madison, WI).

Transformation and Selection in *P. pastoris* X-33 Expression Host. The pGAPZαC plasmids ligated with unmodified cystatin and glycomodified cystatin in-frame DNA sequence were digested with *Bgl*II in the GAP promoter region to linearize the vector and then transformed into *P. pastoris* X-33, respectively, by using the lithium chloride method (*20*). The colonies were selected by plating on Zeocin-YPDS agar plates (20 g/L tryptone, 10 g/L yeast extract, 20 g/L dextrose, 182.2 g/L sorbitol, 20 g/L agar, and 100 μg/mL Zeocin). At least 20 colonies, which were integrated with recombinant and glycomodified cystatins of pGAPZαC plasmids, were selected for investigating the expression of recombinant proteins. Thus, the *Pichia* integrant with the best production of recombinant or glycomodified cystatins was further chosen.

Cultivation of *P. pastoris* X-33 Expression Host and Isolation of Recombinant Cystatin. The chosen *P. pastoris* strains were cultivated with 5 mL of YPDS broth (20 g/L tryptone, 10 g/L yeast extract, 20 g/L dextrose, and 182.2 g/L sorbitol) containing 100 μg/mL Zeocin in a 50 mL flask using a shaking incubator (300 rpm) overnight at 30 °C and then 1 mL of the resulting culture was inoculated into 50 mL of fresh YPD broth (20 g/L tryptone, 10 g/L yeast extract, and 20 g/L dextrose) in a 250 mL flask. It was then cultivated at 30 °C in a shaking incubator (300 rpm) for 4−5 days. The *Pichia* cells were excluded by 10 min of centrifugation at 3000*g*. The supernatant was collected and subjected to further purification.

Purification of Recombinant Chicken Cystatin. The recombinant cystatin was purified according to the method of Chen et al. (*21*) by 40−60% saturated ammonium sulfate, Sephacryl S-100 HR, and Superdex 75 chromatographs, whereas Asn_{106}-glycosylation-modified cystatin was purified by Sephacryl S-100 HR, Con A Sepharose, and Superose 12 chromatographs.

Deglycosylation. The Asn_{106}-glycosylation-modified cystatin was first denatured by heating at 100 °C in the presence of 1% SDS for 10 min. The denatured Asn_{106}-glycosylation-modified cystatin (0.1 mg) was then added to *N*-glycosidase F (5 units; Boehringer Mannheim GmbH, Mannheim, Germany) in 20 mM sodium phosphate buffer (pH 7.2) containing 2% Triton X-100 and 0.2% SDS. The resulting sample was incubated at 37 °C overnight.

Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE analysis was performed according to the method of Laemmli (*22*) using a mini-gel system (SE 260 Hoefer's vertical gel unit, Pharmacia Biotech Inc., San Francisco, CA). The concentration of polyacrylamide gel was 15%. After 30 min of incubation with 1% β-mercaptoethanol at 50 °C, samples were subjected to SDS-PAGE analysis. The staining and destaining were performed according to the method of Neuhoff et al. (*23*). Ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18.4 kDa), lysozyme (14.3 kDa), bovine trypsin inhibitor (6.2 kDa), and insulin (2.3 kDa) were used as markers.

Substrate SDS-PAGE. The substrate SDS-PAGE was performed according to the method of El-Shamei et al. (*24*). A 15% polyacrylamide gel containing 0.1% w/v casein (Merck, Darmstadt, Germany) was employed in this study. About 10−20 μg of sample protein was applied onto each well of the gels. After electrophoretical running, the gels were prewashed with 2.5% Triton X-100 twice for 30 min each time to remove SDS. The resulting gels were incubated with papain (0.01 mg/mL) in a 0.1 M phosphate buffer containing 2 mM cysteine and 1 mM EDTA (pH 6.0) at 37 °C for 60 min. The reaction was stopped by staining solution (mixture of 0.01% Coomassie brilliant blue, 40% methanol, and 10% acetic acid). After destaining with 25% ethanol and 10% acetic acid, the visible intense blue bands were the active cystatin zones.

Freezing Stability. To investigate the influence of Asn_{106}-glycosylation on the freezing stability of cystatin, the wild-type, recombinant, and Asn_{106}-glycosylation-modified cystatins were frozen at −20 °C in distilled water for 14 h and then thawed at 25 °C for 10 h, for six freeze−thawing cycles. During each freeze−thawing process, the remaining activities of each cystatin were determined.

Assay of Enzyme Inhibitory Activity. Papain (EC 3.4.22.2, 2× crystallized; Sigma Chemical Co.) was used for the inhibition assessment. The inhibitory activity of cystatin was assayed by measuring the remaining papain activity using Z-Phe-Arg-MCA (Peptide Institute, Osaka, Japan) as substrate (*25*). The enzyme in 0.2 M sodium phosphate buffer (pH 6.0) containing 4 mM cysteine and 2 mM EDTA with or without cystatin was preincubated at 37 °C for 5 min. The enzyme reaction mixture (0.75 mL) comprised 5 μL of enzyme, 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 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 (Hitachi Instruments Inc., Tokyo, Japan) 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, which was defined as the amount of proteinase that could hydrolyze Z-Phe-Arg-MCA and release 1 nmol of aminomethylcoumarin per minute at 37 °C.

Kinetic Measurements. The inhibitory constants (K_i) were calculated by using the method of Dixon (*26*). Fixed amounts of papain (final 0.005, 0.01, and 0.02 nM for Asn_{106}-glycosylated, wild-type, and recombinant chicken cystatins, respectively) were incubated with appropriate amounts of inhibitors in a 0.1 M sodium phosphate buffer (pH 6.0) containing 2 mM cysteine, 1 mM EDTA, and 0.1% Brij 35 at 40 °C for 3 min. The reaction was started after the addition of various concentrations of substrate (Z-Phe-Arg-MCA; 2, 4, or 10 μM), and the residual activities were measured (*25*). The inhibition constant (K_i) was calculated from the Dixon plots of $1/v$ versus [I].

Protein Concentration Measurements. Protein concentrations were determined according to the dye binding method (*27*) using bovine serum albumin as the standard.

RESULTS

After the pGAPZαC chicken cystatin plasmid had been transformed into the *P. pastoris* X-33 expression host, the expression vector was integrated into genomic DNA due to the existence of the GAP promoter sequence. Because the chicken cystatin gene was under the control of the GAP promoter, a high level of the recombinant chicken cystatin was expressed and secreted into the broth by α-factor preprosequence during shaking cultivation. In both strains (with/without glycosylation-modified mutants), the highest level of cystatin activity (∼6.33 units/mg) was observed after 2 days of shaking cultivation. No significant difference in total cystatin activity between these two strains was obtained after 2 days of shaking cultivation or thereafter (data not shown). According to the growth and cystatin secretion curves (data not shown), the amount of proteins secreted increased up to 4 days of cultivation, whereas the cystatin secretion stopped after 2 days of cultivation. This phenomenon might be due to the death of cells, which increased the amount of proteins in the broth. Other reasons may include the secretion of other proteins such as cysteine proteinases, which react with cystatin or other proteinases that can hydrolyze cystatin during 2−4 days of cultivation. Because no significant increase in cystatin activity was observed during the further cultivation, 2-day cultivation was used in this study.

Table 1. Comparison of the Freezing Activities of Wild-Type, Recombinant, and Asp_{106}-Glycosylation-Modified Chicken Cystatin

	cycle						
	0	1	2	3	4	5	6
wild-type cystatin activity[a] (units)	35.45	32.02	30.04	28.45	26.90	25.02	22.46
relative residual activity (%)	100	90.32	84.73	80.23	75.87	70.56	63.35
recombinant cystatin activity[b] (units)	147.23	138.52	132.76	127.60	101.75	100.73	95.36
relative residual activity (%)	100	94.08	90.17	86.67	69.11	68.42	64.77
glycosylated cystatin activity[b] (units)	156.32	153.02	153.45	150.36	150.76	146.35	146.01
relative residual activity (%)	100	97.89	98.16	96.19	96.44	93.61	93.45

[a] The wild-type chicken cystatin was purchased from Sigma Co. Ltd. and purified again by Superose-12 chromatography. [b] The purified recombinant chicken cystatin and its glycosylated form were expressed in pGAPZαC-cystatin transformed *P. pastoris*.

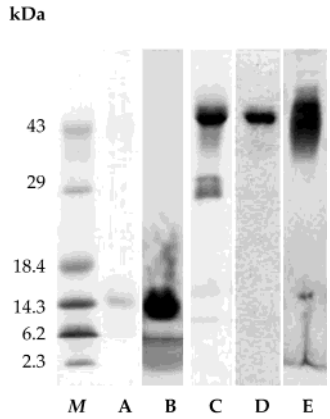

Figure 1. SDS-PAGE and activity staining of recombinant and Asn_{106}-glycosylation-modified cystatins using 15% of polyacrylamide electrophoresis [lane *M*, protein marker; lane A, recombinant cystatin; lanes B and E, activity staining (0.1% casein as substrate) of recombinant and Asn_{106}-glycosylated cystatins; lane C, Sephacryl S-100 HR chromatography of glycosylated cystatin; lane D, glycosylated cystatin].

The Asn_{106}-glycosylation-modified *Pichia* strain, which was integrated with N-Q-^{108}I → N-Q-^{108}T mutant cystatin gene down-strand of the GAP promoter in its chromosome, expressed two recombinant cystatins in the broth. Two forms of recombinant cystatins were separated by Sephacryl S-100 HR gel filtration chromatography. One was polyglycosylated, which amounted to ∼50% of the total recombinant cystatins. The other one was unglycosylated form or glycocystatins with very low degree of polymerization (data not shown).

The nonmodified recombinant cystatin was purified according to the previous study (*21*), whereas the Asn_{106}-glycosylated mutant cystatin was purified to electrophoretical homogeneity by Sephacryl S-100 HR (**Figure 1**, lane C), Con A Sepharose, and Superose 12 (**Figure 1**, lanes D and E) chromatographs. According to N-terminal sequences analysis, the sequences of these two purified recombinant cystatins (non- and glycosylation modification) were as predicted (data not shown). The molecular masses (*M*) of the recombinant chicken cystatin and its Asn_{106}-glycosylated mutant were 14 and 20.5 kDa, estimated by FPLC Superose 12 chromatography, respectively. Both Asn_{106}-glycosylated mutant cystatin and its deglycosylated form were further confirmed by the substrate SDS-PAGE against papain (**Figure 1**, lanes B and E). As indicated in **Figure 1**, the Coomassie brilliant blue stained unhydrolyzed casein band indicated the existence of papain inhibitor, which was the recombinant cystatins.

According to the Dixon plot (data not shown), the curves of recombinant cystatin and Asn_{106}-glycosylated mutant cystatin, obtained from various concentrations of substrate and inhibitors, intersected at a point in quadrant II. These phenomena suggested that the recombinant cystatins with/without glycosylation modification act as competitive inhibitors, which was similar to that of wild-type cystatin. The K_i values for the interaction of papain with wild-type, recombinant, and glycosylation-modified chicken cystatins were 0.05, 0.08, and 9.5 nM, respectively.

From the comparison of the residual activity of recombinant and its Asn_{106}-glycosylation-modified cystatins during six freeze−thawing cycles (freezing at −20 °C for 14 h and thawing at 25 °C for 10 h), there was still 93% of the original activity left in the Asn_{106}-glycosylation-modified cystatin, but only 65 and 63% were left in nonmodified recombinant and wild-type cystatins, respectively (**Table 1**). This phenomenon suggested that the carbohydrate moiety on the Asn_{106}-glycosylation-modified cystatin has a stabilizing effect on the cystatin protein.

DISCUSSION

The three-dimensional structure of chicken cystatin, determined from X-ray and NMR studies, indicates that the shape of the enzyme is wedgelike (*6*, *7*). According to results from several studies (*7*, *28*, *29*), the residues of Q(53), L(54),V(55), S(56), G(57), and W(104), located on the edge of the wedge, are involved in the active site and demonstrated a docking inhibition model against papain. These findings suggested the importance of the protein structure of cystatin to the inhibition mechanism. The carbohydrates are frequently employed to prevent protein denaturation during frozen storage (*30*). Therefore, the site-specific glycosylation of proteins using yeast expression systems might be a new approach to enhance the molecular stability of recombinant protein (*13*−*15*). Because the Asn_{106}−Ile_{108} was not in the active site and exposed to the surface of chicken cystatin (*6*, *7*), Thr_{108} was, therefore, selected to replace Ile_{108}, which would not affect the activity of the recombinant cystatin. This ingenious substitution created an N-glycosylation site at Asn_{106}. According to SDS-PAGE, substrate SDS-PAGE (activity staining) (**Figure 1**, lane D), and K_i values, N-Q-^{108}I → N-Q-^{108}T modification can express glycosylated cystatin in *P. pastoris* X-33, and the carbohydrate moiety did not seriously affect its papain inhibition ability as expected. The estimated *M* of glycosylated cystatin, 20.5 kDa on Superose 12 chromatography, coincided with the sum of protein moiety (14 kDa) and polysaccharide moiety (40 DP, ∼6.5 kDa calculated from mannose). However, the slower migration of glycosylated cystatin on SDS-PAGE revealed a larger *M* than 50 kDa. This might be due to the existence of a polysaccharide moiety, which consequently affected the migration of glycoprotein on SDS-PAGE. When the polysaccharide moiety was removed, the migration of deglycosylated cystatin on SDS-PAGE was similar to that of nonmodified cystatin (data not shown).

Although the physiological properties of glycosylation are still not completely understood, the secretion of proteins and

induction of glycosylation at asparagine sites with recognition sequences of Asn-X-Ser/Thr in yeasts and the role of the carbohydrate moiety of glycoproteins have been well documented (*31*, *32*). They include the effect of glycosylation on protein solubility, proteolytic degradation, and thermal stability of the protein (*13*−*15*). N- and O-linked oligosaccharides were also found to facilitate the protein folding, subunit assembly, and secretion of some glycoproteins (*15*, *33*). According to some studies, the oligosaccharides of glycoproteins have a significant effect on their specific activity (*34*). In this study, the presence of oligosaccharide substantially enhanced the stability of glycosylation-modified cystatin against freeze denaturation. This result suggested that modification of Asn_{106}−Ile_{108} to Asn_{106}−Thr_{108} successfully induced the glycosylation on Asn_{106} (**Figure 1**, lane D), which consequently confers the glycosylation-modified recombinant cystatin upon freezing stability.

Compared with the K_i of recombinant cystatin without glycosylation modification and wild-type chicken cystatin against papain, that of Asn_{106}−Thr_{108} glycosylation-modified cystatin was ∼190-fold that of the wild-type one. This result suggested that the polysaccharide chain on Asn_{106}−Thr_{108} glycosylation-modified cystatin might hinder the cystatin from the collision with binding areas of papain, because the glycosylation-modified point was near the active site according to the X-ray crystal structure of chicken egg white cystatin (*7*). On the basis of a review of the literature thus far published, the present study is the first report using glycosylation modification to enhance the freezing stability of recombinant proteins. Although the glycosylation modification has negligible effect on protein structure, it substantially improved the freezing stability of the recombinant cystatin. This might be due to the existence of a carbohydrate moiety on the protein, which consequently provides a cushion effect among proteins against the formation of interactions such as covalent bonds and hydrophobic and hydrophilic interactions during freezing and subsequent storage. This might also be due to the existence of a carbohydrate moiety, which consequently increased the hydrogen bonds and absorbed more water to prevent the exposure of functional groups during freezing and subsequent storage and thus resulted in a decrease in the formation of interactions among proteins.

According to the data obtained from this study, although the Asn_{106}−Thr_{108} glycosylation-modified recombinant chicken cystatin had lower inhibition ability, compared with wild-type and non-glycosylation-modified cystatins, it is still a strong inhibitor (K_i = 9.5 nM) for papain or even for other cysteine proteinases. Furthermore, the expression system developed in this study is useful and economical in producing the freezing-tolerant glycosylation-modified recombinant cystatin for industrial application.

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