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Overexpression and Functional Characterization of a Serine Carboxypeptidase Inhibitor (I^C) from *Saccharomyces cerevisiae*

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Carboxypeptidase Y (CPY) inhibitor, I^C, a cytoplasmic inhibitor of vacuolar proteinases in yeast, *Saccharomyces cerevisiae*, was purified by means of a high-level expression system using a proteinase-deficient strain, BJ2168, and an expression vector with the promoter *GAL1*. The purified I^C exists as a monomeric β-protein in solution with a molecular weight of 24,398.4 as determined by gel filtration chromatography, MALDI-TOF mass spectrometry, and far-UV CD spectroscopy. The acetylated N-terminal methionine residue is the sole posttranslational modification. I^C specifically inhibits both the peptidase and anilidase activities of CPY with inhibitor constants (K_i) of approximately 1.0×10^{-9} M. The chemical modification of I^C with sulfhydryl reagents indicated that it lacks disulfide bonds and has two free SH groups, which are responsible, not for the inhibitory function, but, apparently, for the folding of the overall structure. The formation of a complex of I^C with CPY was highly specific, as evidenced by no detectable interaction with pro-CPY. Chemical modification studies of the CPY-I^C complex with specific reagents demonstrated that the catalytic Ser146 and S1 substrate-binding site of CPY are covered in the complex.

Key words: carboxypeptidase inhibitor, carboxypeptidase Y, I^C, overexpression, proteinase-inhibitor complex.

Endogenous protein inhibitors of lysosomal and vacuolar proteinases have been discovered in the cytoplasm of various organisms, although the roles of these inhibitors in the regulation of cognate proteinases remain unclear. The cytoplasmic inhibitors of lysosomal cysteine proteinases, family I cystatins, are widely distributed in animal cells and well characterized in terms of their biochemical properties, amino acid sequences, and crystal structures. These protein inhibitors appear to be relatively small proteins (M_r 11,000–13,000) and lack disulfide bonds (*1–3*).

Three cytoplasmic inhibitors of vacuolar proteinases have been identified to date in the yeast, *Saccharomyces cerevisiae*: proteinase A inhibitor, I^A, proteinase B inhibitor, I^B, and carboxypeptidase Y (CPY) inhibitor, I^C (*4–8*). The former two inhibitors, I^A and I^B, are small proteins composed of about 70 amino acid residues, and contain no cysteine residues (*9, 10*). The mechanisms by which I^A and I^B function as inhibitors have been reported by systematic mutational analyses (*11, 12*). Furthermore, X-ray crystallographic studies of the complex of I^A with proteinase A revealed that the N-terminal half of the inhibitor, which has no intrinsic secondary structure in solution, is converted to an α-helical conformation to mask the active site of proteinase A (*13*). From a biological point of view, I^B seemingly promotes the fusion of yeast vacuoles via the formation of a heterodimer with thioredoxin, although the inhibitory activity is not related to this proposed function of I^B (*14, 15*).

On the other hand, precise information on the functional and structural properties of I^C are not currently available, except for some physical and chemical properties, such as its molecular weight of 23,400–24,000 and the fact that the N-terminal amino acid is acetylated (*5, 16*). Recently, the partial amino acid sequence of I^C was determined and it has been suggested that I^C is encoded by the *TFS1* gene, which had been identified with a dosage-dependent suppressor of *cdc25* mutations (*17, 18*). The *TFS1* gene product shows homology to "21–23 kDa lipid binding proteins", which are putatively involved in signal mechanisms during cell growth and maturation (*17–19*). In yeast cells, the nitrogen- and glucose- specific signaling pathways are controlled by the *CDC25* gene product (*20*), while the level of vacuolar proteinase activities changes during cell growth and maturation depending on nutritional conditions (*21, 22*). The homology of I^C to the lipid binding proteins may reflect the relationship between the *CDC25*-controlled nutrient signaling pathway and the regulation of proteolysis in a vacuole. In the present study, a high-level expression system for I^C was constructed, and its biochemical properties were investigated in an attempt to obtain insight into the inhibition mechanism of this protein.

MATERIALS AND METHODS

Materials—CPY was purified as previously described (*23*). Synthetic oligonucleotides were obtained from Japan

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Abbreviations: Ac, acetyl; BTPNA, *N*-benzoyl-L-tyrosine-*p*-nitroanilide; CBZ, benzyloxycarbonyl; CPY, carboxypeptidase Y; DMF, *N,N*-dimethylformamide; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); I^A, yeast proteinase A inhibitor; I^B, yeast proteinase B inhibitor; I^C, carboxypeptidase Y inhibitor; PCMB, *p*-chloromercuribenzoic acid; PMSF, phenylmethylsulfonyl fluoride.

Bio Services, Saitama. Butyl Toyopearl 650S was from Tosoh, Tokyo. Superdex 75 and Superdex 200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. A sequencing grade of trypsin was obtained from Promega Corporation, Madison, WI, USA. The restriction endonucleases were from Toyobo, Tokyo. All other chemicals were of reagent grade and were obtained locally.

Strains and Plasmids—The plasmid pTSY3 containing the *PRC1* gene coding for CPY and *Saccharomyces cerevisiae* SEY2202 (*MATa Δprc1::(LEU2) leu2-3, 112 ura3-52 his4-519*), which was used as the host for the expression of CPY, were kindly provided by Dr. Klaus Breddam, the Carlsberg Laboratory, Copenhagen, Denmark. *Saccharomyces cerevisiae* BJ2168 (*MATa prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 gal2*) was used as the host for the expression of pro-CPY and I^C in order to avoid the degradation of these proteins by intracellular proteinases (*24*). Plasmid pYES2 was obtained from Invitrogen Corporation, San Diego, CA, USA

Construction of an Expression Plasmid for I^C—The *TFS1* gene encoding I^C was amplified from yeast genomic DNA using two oligonucleotides: 5′-GGG GTA CCC CGA TGA ACC AAG CAA TAG-3′ and 5′-GGG GTA CCC CGC TAT TTC GTT TCC GC-3′. The expression plasmid for I^C, pYTF1, was produced by ligating the DNA fragment into pYES2. The 670 bp fragment containing the *TFS1* gene was excised by *Kpn*I digestion and inserted downstream of the *GAL1* promoter in pYES2. The DNA sequence of the region containing the *TFS1* gene was determined using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Yeast Transformation—*Saccharomyces cerevisiae* strain BJ2168 was transformed with the expression vector pYTF1, using a EZ-Yeast Transformation Kit (BIO 101, Vista, USA). Ura^+ transformants were selected by plating on selective medium (0.67% yeast nitrogen base without amino acids, 2.0% glucose, 30 μg/ml Leu, and 20 μg/ml Trp).

Expression and Purification of I^C—A single colony of Ura^+ transformants was grown in the selective medium at 28°C. The harvested cells were suspended in the nutrient me-dium (2 % galactose, 1% yeast extract, 2% Bacto peptone) and grown at 28°C.

The collected cells were suspended in 0.1 M sodium phosphate (pH 7.0) containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 1 μg/ml pepstatin A, and subjected to five cycles of freeze-thawing by sequentially immersing the suspension in a liquid nitrogen bath and a water bath at 40°C. Solid ammonium sulfate was gradually added to the combined supernatant under stirring to a concentration of approximately 1.0 M. The mixture was loaded onto a Butyl-Toyopearl 650S column (30 × 60 mm) equilibrated with 1.0 M ammonium sulfate in 0.1 M sodium phosphate (pH 7.0) containing 1 mM EDTA and 1 mM DTT. I^C was eluted with 0.5 M ammonium sulfate in 0.1 M sodium phosphate (pH 7.0) containing 1 mM EDTA and 1 mM DTT. The pooled fraction of I^C was concentrated by ultrafiltration, and applied to a Superdex 200 column (10 × 300 mm) equilibrated with 0.1 M sodium phosphate (pH 7.0) containing 1 mM EDTA, 1mM DTT, and 0.15 M NaCl. I^C was eluted with the equilibrating buffer and concentrated by ultrafiltration. The concentrated sample was again chromatographed on a Superdex 75 column (10 × 300 mm) according to the typical procedure used for chromatography on a Superdex 200 column.

Assay of the Inhibitory Activity of I^C—The inhibitory activity of I^C was determined as the residual activity of CPY with respect to the hydrolysis of 0.3 mM BTPNA in 0.1 M so-dium phosphate (pH 7.0) containing 10% DMF (*23*). To determine the inhibitory unit, CPY (2 μg) was mixed with various quantities of I^C in 0.1 M sodium phosphate (pH 7.0), and incubated at 25°C for 10 min. One unit of the inhibitory activity is defined herein as the quantity of I^C required to inhibit 50% of the CPY activity for BTPNA.

Protein Concentration—Protein concentrations were determined by the bicinchoninic acid method (*25*) using bovine serum albumin as the standard.

Gel Filtration—For determining the molecular weight of I^C and the CPY-I^C complex, gel filtration chromatography was performed with a column (10 × 300 mm) of Superdex 75 calibrated with the following reference proteins: IgG (M_r 160,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (31,000), and RNase A (13,700). Equilibration and elution were carried out with 0.1 M sodium phosphate (pH 7.0) containing 1 mM EDTA, 1 mM DTT, and 0.15 M NaCl at a flow rate of 0.5 ml/min. To investigate the nature of complex formation between I^C and CPY or between I^C and pro-CPY, CPY (100 μg, 11 μM final concentration) or pro-CPY (113 μg, 11 μM) was mixed with I^C (0–80 μg, 0–22 μM) and subjected to Superdex 75 chromatography.

Mass Spectrometry—The molecular masses of I^C and the peptide fragments obtained by tryptic digestion were determined by MALDI-TOF mass spectrometry using a Voyager RP mass spectrometer (Applied Biosystems). Samples were prepared as follow: I^C (3 μM final concentration), which had been previously desalted by reverse-phase chromatography, was added to a mixture of 33% acetonitrile (v/v) containing 7 mg/ml 3,5-dimethoxy-4-hydroxy cinnamic acid (used as a matrix) and 0.2% trifluoroacetic acid. To fragment I^C, trypsin (200 ng) was mixed with 10 μg of I^C in 50 mM NH_4HCO_3 (pH 7.8), and the solution was incubated at 37°C for 3 h. The peptides were desalted on ZipTip C18 (Millipore, Bedford, MA, USA) and mixed with 10 mg/ml α-cyano-4-hydroxycinnamic acid in 60% acetonitrile (v/v) containing 0.3% trifluoroacetic acid. Data were calibrated with standard proteins or peptides containing bovine serum albumin, bovine trypsinogen, horse apomyoglobin, bovine insulin B chain (oxidized), and human angiotensin II.

Circular Dichroism Spectroscopy—CD spectra in the region 200 to 260 nm were measured in a cell with an optical pathlength of 0.1 cm at 25°C with a Jasco J-720W spectropolarimeter in 10 mM sodium phosphate, pH 7.0 containing 1 mM EDTA and 1 mM DTT. The concentrations of I^C, CPY, and CPY-I^C complex were 8.2 μM. Baseline-corrected CD spectra were deconvoluted with the CDFIT program (*26*).

Chemical Modification of I^C with Sulfhydryl Reagents—Free sulfhydryl groups of I^C were determined by titration with DTNB and PCMB under denaturing and non-denaturing conditions as follows. Five hundred micromolar DTNB was mixed with 20 μM I^C in 20 mM sodium phosphate (pH 8.0) containing 1 mM EDTA in the absence or presence of 0.5% SDS and incubated at 25°C for 0–24 h. SH groups were determined assuming an extinction coefficient of 13,380 $M^{-1}\cdot cm^{-1}$ at 412 nm (*27*). Forty micromolar PCMB

was mixed with 20 mM I^C in 50 mM sodium phosphate (pH 7.0) in the absence or presence of 0.5% SDS and incubated at 25°C for 0–24 h. SH groups were determined assuming an extinction coefficient of 7,600 $M^{-1}\cdot cm^{-1}$ at 255 nm (*28*). The residual inhibitory activity of modified I^C after sulfhydryl modification was measured in 0.1 M sodium phosphate (pH 7.0) with a mixture of 32.8 nM CPY, 65.6 nM modified I^C, and 0.3 mM BTPNA.

Determination of Inhibitory Constants—Inhibitor constants (K_i) of I^C toward CPY were determined for the anilidase (*23*) and peptidase activities. CPY (32.8 nM) was mixed with various concentrations of I^C (0–164 nM) in 0.1 M sodium phosphate buffer (pH 7.0) containing 0.3 mM BTPNA and 10% DMF or in 50 mM bis-Tris (pH 6.5) containing 1.0 mM CBZ-Ala-Phe-OH and 1 mM EDTA and incubated at 25°C. The initial hydrolytic rates of CBZ-Ala-Phe-OH were determined by quantitating the amount of phenylalanine released on a JEOL JLC-500/V amino acid analyzer (JEOL, Tokyo). The apparent inhibitor constants, K_i(app), were estimated using the following equation:

$$[I]_0/\{1 - (V_i/V_0)\} = \{K_i(\text{app})/(V_i/V_0)\} + [E]_0 \quad (1)$$

where V_0 and V_i are the CPY activity in the absence and presence of the inhibitor, respectively. A plot of $[I]_0/\{1 - (V_i/V_0)\}$ against $1/(V_i/V_0)$ gave a slope equal to K_i(app) (*29*). Since I^C is a competitive inhibitor (*30*), the intrinsic inhibitor constants, K_i, were calculated from the following equation:

$$K_i = K_i(\text{app})/(1 + [S]/K_m) \quad (2)$$

where the K_m values used were 0.33 and 1.1 mM for BTPNA and CBZ-Ala-Phe-OH, respectively.

Reactivity of PMSF and PCMB with CPY-I^C Complex—A twentyfold molar excess of PMSF or PCMB, specific reagents for the catalytic center Ser146 and the S1 subsite residue Cys341 of CPY, respectively (*31*), was mixed with 50 mM bis-Tris (pH 6.5) containing 2 μM CPY-I^C complex. For the reaction with PCMB, I^C previously modified with the reagent, which fully maintains its inhibitory activity, was used to form the complex with CPY after removal of the excess reagent by gel filtration chromatography on Superdex 75. Residual CPY activities during the reaction were measured in 0.1 M sodium phosphate (pH 7.0) containing 0.3 mM BTPNA, 1 mM EDTA, 10% DMF, and 1.2 M guanidinium chloride, where the CPY-I^C complex exhibits CPY activity equivalent to that of free CPY (*18*).

RESULTS

Expression and Purification of I^C—The expression level of I^C was increased significantly in the stationary growth phase and suppressed in the logarithmic growth phase. A freeze-thawing method for the extraction of I^C resulted in a higher yield than other extraction methods such as heat-treatment (*7*), autolysis with chloroform (*23*), and cell disruption with glass beads (see lane 2 in Fig. 1). In the final purification, 29 mg of I^C was obtained from 100 g of yeast wet cells contained in 2 liters of cultured medium with an overall yield of 36%. The homogeneity of I^C in this preparation was confirmed by SDS-PAGE analysis (Fig. 1).

Molecular Properties of I^C—The molecular weight of I^C was estimated to be 25,000 by Superdex 75 gel filtration chromatography (Fig. 2), in good agreement with the putative molecular weight, which was calculated to be 24,357.3 from the predicted amino acid sequence of the *TFS1* gene product (*17, 18*). This result shows that I^C is present in a monomeric form in solution. MALDI-TOF mass spectrometry revealed the molecular weight of I^C to be 24398.4, which is larger than the putative molecular weight by 41.1 (Fig. 2).

Mass spectrometric analyses of the molecular masses of the peptide fragments obtained by tryptic digestion of I^C are summarized in Table I. The masses of eleven peptides corresponding to residue numbers 18 to 219 are consistent

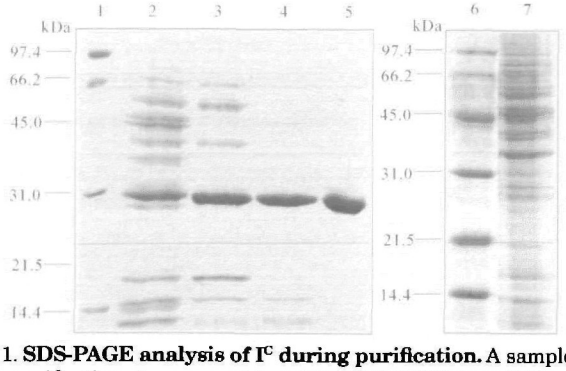

Fig. 1. **SDS-PAGE analysis of I^C during purification.** A sample at each purification step was subjected to SDS-PAGE in a 15% polyacrylamide gel. Lane 1, molecular size markers (phosphorylase *b* for 97.4 kDa, bovine serum albumin for 66.2 kDa, ovalbumin for 45.0 kDa, carbonic anhydrase for 31.0 kDa, soybean trypsin inhibitor for 21.5 kDa, lysozyme for 14.4 kDa); lane 2, the crude extract prepared by freeze-thawing yeast cells; lanes 3–5, fractions containing inhibitory activity from butyl-Toyopearl 650S, Superdex 200 gel filtration, and Superdex 75 gel filtration chromatographies, respectively; lane 6, molecular size markers; lane 7, the crude extract from untransformed BJ2168 cells.

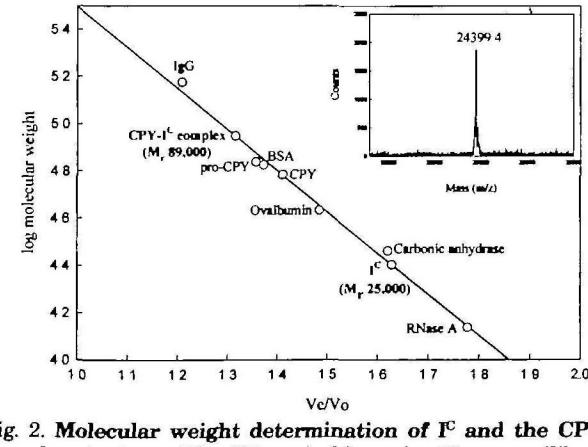

Fig. 2. **Molecular weight determination of I^C and the CPY-I^C complex.** A column (10 × 300 mm) of Superdex 75 was equilibrated with 0.1 M sodium phosphate (pH 7.0) containing 1 mM EDTA, 1 mM DTT, and 0.15 M NaCl. I^C (40 μg), CPY-I^C complex (140 μg), and the reference proteins (50–100 μg) were eluted with the same buffer at a flow rate of 0.5 ml/min with monitoring by the absorbance at 280 nm. The void volume (V_0) was estimated to be 7.77 ml with blue dextran. The insert shows the mass spectrum in which a mixture of the purified I^C (3 μM), 3,5-dimethoxy-4-hydroxy cinnamic acid (7 mg/ml), 33% acetonitrile, and 0.2% trifluoroacetic acid was loaded on a sample plate and the calibration was done with bovine serum albumin, trypsinogen, and apomyoglobin. The molecular mass of I^C is shown in the figure.

TABLE I. **Masses of tryptic fragments from I^C.**

Residue number[a]	Sequence	Observed mass (*m/z*)	Predicted mass (*m/z*)
1–16	NH_2-MNQAIDFAQASIDSYK	—	1,801.84
1–16	Ac-MNQAIDFAQASIDSYK	1,843.83	1,843.85
1–17	NH_2-MNQAIDFAQASIDSYKK	—	1,929.94
1–17	Ac-MNQAIDFAQASIDSYKK	1,971.96	1,971.95
18–56	HGILEDVIHDTSFQPSGILAVEYSSSAPVAMGNTLPTEK	4,113.79	4,113.62
59–69	SKPQFQFTFNK	1,371.63	1,371.71
74–101	SVPQANAYVPQDDDLFTLVMTDPDAPSK	3,036.22	3,036.36
74–105	SVPQANAYVPQDDDLFTLVMTDPDAPSKTDHK	3,517.98	3,517.87
119–141	LLNEATHETSGATEFFASEFNTK	2,544.29	2,544.19
142–156	GSNTLIEYMGPAPPK	1,574.68	1,574.79
157–162	GSGPHR	610.28	610.31
163–169	YVFLLYK	945.45	945.55
182–199	IKDRPNWGYGTPATGVGK	1,917.00	1,917.00
184–199	DRPNWGYGTPATGVGK	1,675.76	1,675.82
203–219	ENNLQLVASNFFYAETK-COOH	1,987.98	1,987.98

[a]Residue number is obtained from the predicted amino acid sequence of the *TFS1* gene product.

TABLE II. **Secondary structure contents, as estimated from far-UV CD spectra.**[a]

	α-Helix (%)	β-Sheet (%)	Random coil (%)
I^C	1.87	79.7	18.4
CPY	12.9	53.4	33.7
CPY-I^C complex	8.03	54.9	37.0
Sum of CPY and I^C	9.13	62.4	28.5

[a]CDFIT program was used for the deconvolution of the CD spectra.

with the masses of the predicted peptide fragments. However, the masses of two N-terminal peptides, 1,843.83 for residues 1–16 and 1,971.96 for residues 1–17, are larger than those of the predicted N-terminal fragments by 41.99 and 42.02, respectively, values that are nearly identical to the weight of an acetyl group, 42.01. Thus, we conclude that the acetylation of the N-terminal methionine residue is the sole posttranslational modification in I^C.

Alpha-helix, β-sheet, and random structure in the I^C molecule were calculated from the far-UV CD spectrum and are summarized in Table II. The results show that I^C is a β-type protein in which β-structure is the predominant structural element.

Two moles of SH group per mole I^C were detected by DTNB and PCMB (Fig. 3B). These cysteine residues have been identified as Cys110 and Cys115 (*17, 18*). Thus, I^C contains no disulfide bonds. The reaction rates of DTNB and PCMB with native I^C were slow: Free SH groups after 24 h were estimated to be 1.2 mol and 1.75 mol per mol I^C with DTNB and PCMB, respectively (Fig. 3A).

Inhibitory Properties of I^C—The anilidase activity for BTPNA and peptidase activity for CBZ-Ala-Phe-OH of CPY were similarly inhibited by I^C: an equivalent molar concentration of I^C inhibited 80% of each activity of CPY and a 5-fold molar excess of I^C inhibited the activities by more than 95% (Fig. 4). Based on these data, the apparent inhibitor constants, K_i(app), were calculated to be 2.6×10^{-9} and 2.0×10^{-9} M for BTPNA and CBZ-Ala-Phe-OH hydrolyses, respectively (see Eq. 1). These values are not inconsistent with that for Ac-Tyr-O-Et hydrolysis, 2.5×10^{-9} M (*16*). The intrinsic inhibitor constants, K_i, were estimated to be 1.4×10^{-9} and 1.0×10^{-9} M for BTPNA and CBZ-Ala-Phe-OH hydrolyses, respectively (see Eq. 2).

Incubation with PCMB under non-denaturing conditions

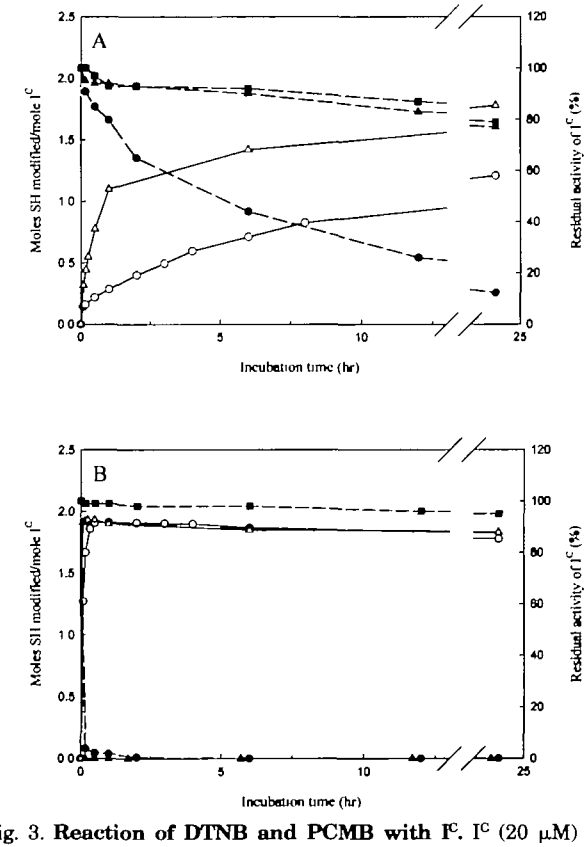

Fig. 3. **Reaction of DTNB and PCMB with I^C.** I^C (20 μM) was mixed with DTNB (500 μM) in 20 mM sodium phosphate (pH 8.0) containing 1 mM EDTA, or PCMB (40 μM) in 50 mM sodium phosphate (pH 7.0), in the absence (A) or presence (B) of 0.5 % SDS. The mixture was incubated at 25°C and an aliquot was withdrawn to determine the degree of SH modification and inhibitory activity. See "MATERIALS AND METHODS" for other details. Open circles, moles SH modified in the DTNB reaction; open triangles, moles SH modified in the PCMB reaction; closed circles, residual activity in the DTNB reaction; closed triangles, residual activity in the PCMB reaction; closed squares, residual activity of unmodified I^C.

for 24 h resulted in the modification of more than 80% of the SH groups without affecting the inhibitory activity (Fig. 3A). On the other hand, modification of the SH groups with DTNB resulted in a gradual decrease in the residual activ-

ity by up to 20% (Fig. 3A). Therefore, the influence of DTNB-modification on the inhibitory activity of I^C is substantially different from that of PCMB-modification. After incubation in 0.5% SDS (for 0–24 h), the inhibitory activity of I^C was fully recovered as a result of the dilution of the denaturant, but the recovery of activity disappeared after incubation with PCMB or DTNB in 0.5% SDS (Fig. 3B).

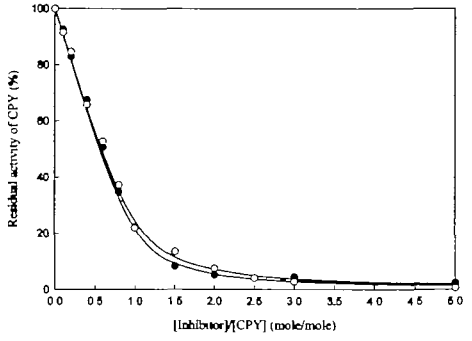

Fig. 4. **Stoichiometry of the CPY inhibition by I^C.** CPY (32.8 nM) was mixed with various amounts of I^C (0–164 nM) and the residual activity of CPY was assayed using BTPNA or CBZ-Phe-Ala-OH as the substrate. Open circles, BTPNA activity; closed circles, CBZ-Phe-Ala-OH activity. Curves represent the weighted least-squares fit to Eq. 1. See "MATERIALS AND METHODS" for other details.

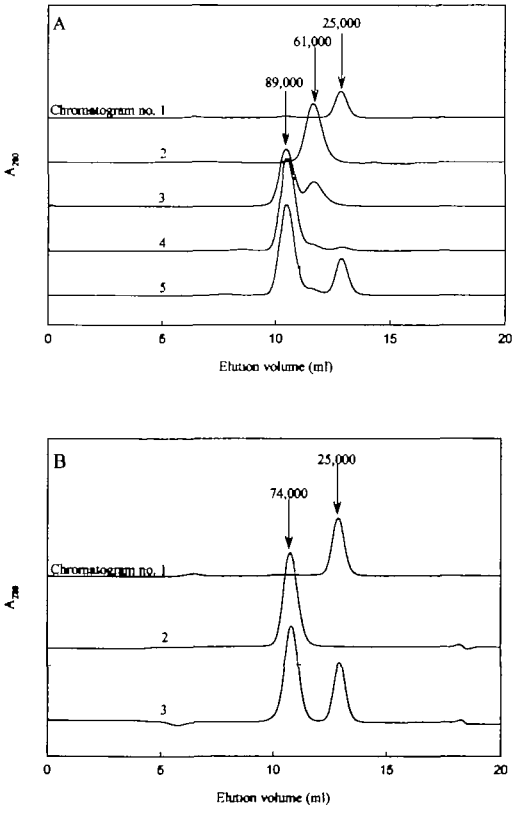

Fig. 5. **Gel filtration analysis of I^C and CPY or I^C and pro-CPY mixtures.** An I^C and CPY mixture or I^C and pro-CPY mixture was applied to a Superdex 75 column (10 × 300 mm). The estimated molecular weight of each peak is shown in the figures. (A) Chromatogram no. 1, 11 μM I^C; no. 2, 11 μM CPY; no. 3, 5.5 μM I^C and 11 μM CPY mixture; no. 4, 11 μM I^C and 11 μM CPY mixture; no. 5, 22 μM I^C and 11 μM CPY mixture. (B) Chromatogram no. 1, 22 μM I^C; no. 2, 11 μM proCPY; no. 3, 22 μM I^C and 11 μM proCPY mixture. See Fig. 2 for other details.

Properties of the Complex of I^C with CPY—The stoichiometry of complex formation of I^C and CPY or pro-CPY was analyzed by Superdex 75 gel filtration chromatography (Fig. 5A). The addition of increasing amounts of I^C (0–11 μM) to a solution containing a fixed concentration of CPY (11 μM) produced a new peak of M_r 89,000, which increased proportionally with the addition of I^C accompanied by an inverse decrease in the M_r 61,000 peak (chromatogram no. 2–4 in Fig. 5A). The M_r 89,000 peak alone was obtained with an equimolar mixture of I^C and CPY, while the I^C (M_r 25,000) peak appeared in mixtures containing a 2.0-fold molar excess of I^C over CPY in addition to the M_r 89,000 peak (chromatogram nos. 4 and 5 in Fig. 5A). These results clearly demonstrate that I^C is involved in the formation of an equimolecular complex of I^C and CPY with M_r 89,000 (Figs. 3 and 5A). On the other hand, pro-CPY, which is an inactive precursor of CPY with a pro region comprising 91 amino acids at the N-terminus, formed no complex with I^C (Fig. 5B): A 2.0-fold molar excess of I^C over pro-CPY produced two peaks for pro-CPY (M_r 74,000) and I^C. The formation of a complex of I^C with CPY but not pro-CPY was also demonstrated by non-denaturing PAGE analysis (data not shown).

The secondary structure contents of the CPY-I^C complex were estimated as 8% α-helix, 55% β-sheet, and 37% random coil from the far-UV CD spectrum of the complex (Table II). Compared with the sum of CPY and I^C, the spectrum of the complex was altered in the range of 205–225 nm to some extent, and the content of β-sheet decreased by 7.5% with an 8.5% increase in random coil content.

In order to evaluate the reactivity of the active site residues of CPY in forming the CPY-I^C complex, the reactivity of specific reagents, PMSF and PCMB, toward the CPY portion of the complex was analyzed. The CPY portion of the CPY-I^C complex was only slightly inactivated within 20 min by either reagent: The rates of inactivation were large-

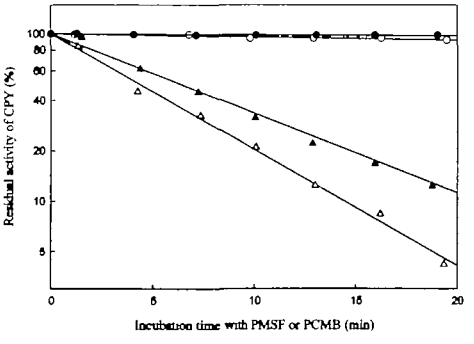

Fig. 6. **Effect of PMSF and PCMB on the CPY-I^C complex.** A 20-fold molar excess of PMSF or PCMB was mixed with 2 μM CPY-I^C complex or CPY, and the mixture was incubated at 25°C. Residual activity for BTPNA was determined in 0.1 M sodium phosphate (pH 7.0) containing 1.2 M guanidinium chloride at the times indicated. The activity before incubation was taken as 100%. Open circles, PMSF reaction with the CPY-I^C complex; closed circles, PCMB reaction with the CPY-I^C complex; open triangles, PMSF reaction with CPY; closed triangles, PCMB reaction with CPY. The second order reaction rates, k_{2nd}, for the inactivation of the CPY part of CPY-I^C complex were 0.22 $min^{-1}·mM^{-1}$ for PMSF and 0.037 $min^{-1}·mM^{-1}$ for PCMB. Those of free CPY were 8.0 $min^{-1}·mM^{-1}$ for PMSF and 2.7 $min^{-1}·mM^{-1}$ for PCMB.

ly decreased as compared with that of free CPY with second order reaction rates, k_{2nd}, of 0.22 $min^{-1}\cdot mM^{-1}$ for PMSF and 0.037 $min^{-1}\cdot mM^{-1}$ for PCMB (Fig. 6). These results indicate that Ser146 in the catalytic triad and Cys341 at the S1 subsite of CPY, which is thought to interact with the catalytic His397 in the transition state (*32*), are covered as a result of complex formation with I^C.

DISCUSSION

A high expression system for I^C was constructed using a vector containing the promoter *GAL1*, and the proteinase A, B and a CPY-deficient strain of *Saccharomyces cerevisiae*, BJ2168 (Fig. 1). The yield of purified I^C was increased by approximately 300-fold compared to that of the wild type yeast (*5*). I^C is a heat-stable protein similar to proteinase A and B inhibitors, I^A and I^B (*6–8*), and has been purified by the homogenization of yeast cells at 100°C (*7*) or precipitation of contaminated proteins at 80°C (*18*). The present method eliminates possible adverse effects on the structure and/or function of the protein caused by such vigorous treatments.

Chemical modification of native and denatured I^C by DTNB and PCMB revealed that I^C contains no disulfide bonds but two free SH groups, which are reactive with the reagents even in the native form. However, modification with PCMB under non-denaturing conditions had no effect on the inhibitory activity of I^C, while modification with DTNB largely reduced the inhibitory activity. On gel filtration chromatography, the PCMB-modified I^C eluted at the same position as native I^C, but the DTNB-modified I^C adsorbed irreversibly to the column (data not shown). These results indicate that the two SH groups are located some distance from the reactive site of I^C, but that modification by DTNB may convert native I^C into an inactive form with an accompanying overall conformational change. This may be due to the formation of a new intramolecular disulfide bond (Cys110–Cys115) *via* secondary SH-disulfide exchange reactions, as has been previously observed in the case of D-glyceraldehyde-3-phosphate dehydrogenase (*33*). The DTNB- and PCMB-modifications under denaturing conditions demonstrate that I^C is immediately refolded upon dilution of the denaturant, but the refolding process is perturbed by modifications by both reagents at the free SH groups. Thus, the free SH groups of I^C may contribute, not its inhibitory properties, but, rather, to the folding and maintaining of the overall structure.

An equimolecular complex of I^C with CPY was formed, as observed by gel filtration chromatography and native-PAGE, while no interaction of I^C with the inactive precursor, pro-CPY, was found by either analytical method. The active-site cleft of pro-CPY is exposed to the solvent but appears to be converted into an inactive conformation due to the allosteric effect of the propeptide region (*34*). Thus, I^C strictly distinguishes the active-site cleft of CPY from that of pro-CPY.

The different reactivities of PMSF and PCMB, specific inhibitors of the catalytic Ser146 and Cys341 at the S1 binding pocket of CPY, respectively, toward the CPY portion of the CPY-I^C complex provides information concerning the binding mode of CPY and I^C: A significant decrease in the reaction rates of PMSF and PCMB toward the complex confirms that Ser146 and Cys341 of CPY are masked by the reactive site of I^C in the complex. This is consistent with a previous study showing that I^C competitively inhibits the peptidase activity of CPY (*30*). Considering that I^C was isolated from the CPY-I^C complex with its C-terminal portion intact (*18*), the possible mechanism of CPY inhibition by I^C must be analogous to the substrate-like direct blockage inhibition of subtilisin BPN′ by *Streptomymyces* subtilisin inhibitor (*35*), and different from the indirect blockage inhibition of papain-like cysteine proteinase by cystatin (*1–3*) or the stopper-like inhibition of carboxypeptidase A by potato carboxypeptidase inhibitor (*36*).

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