J. Biochem. 127, 427–433 (2000)

N-Glycans Protect Proteins from Protease Digestion through Their Binding Affinities for Aromatic Amino Acid Residues[1]

Tomoko Nishiyama, Naoya Kimura, Yuki Jitsuhara, Makoto Uchida, Fumiko Ochi, and Haruki Yamaguchi[2]

Department of Applied Biological Chemistry, College of Agriculture, Osaka Prefecture University, Gakuen-cho 1-1, Sakai, Osaka 599-8531

Received November 12, 1999; accepted December 14, 1999

It was previously revealed [Yamaguchi, H., Nishiyama, T., and Uchida, M. (1999) *J. Biochem.* 126, 261–265] that *N*-glycans of both the high-mannose and complex types have binding affinity for aromatic amino acid residues. This study shows that free *N*-glycans protect proteins from protease digestion through their binding affinities for the aromatic amino acid residues exposed on protein molecules. Protease digestion of bovine pancreatic RNase A and bovine α-lactalbumin was depressed in solutions (1 mM or so) of free *N*-glycans of both the high-mannose and complex types. The increasing order of the protective effects of the *N*-glycans paralleled that of their affinities for aromatic amino acid residues; and the presence of aromatic amino acids practically abolished the protective effects of the *N*-glycans. The *N*-glycans also depressed the protease digestion of metallothionein, an aromatic amino acid-free protein, in agreement with the observation that the *N*-glycans also interact with the solvent-exposed aromatic amino acid residues of the proteases. Thus it seems probable that the *N*-glycans protect proteins from protease digestion by steric hindrance attributable to their binding affinity for the solvent-exposed aromatic amino acid residues of both substrate proteins and proteases.

Key words: *N*-glycan, *N*-glycan function, *N*-glycan–protein interaction, glycoprotein, protease.

In recent years, *N*-glycans have been found to be closely related to protein conformation. It has become apparent that the intramolecular *N*-glycans are directly involved in the stabilization of protein conformation (*1–6*). On the other hand, we recently revealed that the intramolecular high-mannose type *N*-glycans directly promote the oxidative refolding of reductively denatured pancreatic RNase B (*7, 8*). Such a chaperone-like function was also observed for extramolecular free *N*-glycans of the complex as well as the high-mannose type (*8, 9*). These studies also provided evidence to suggest that free *N*-glycans of both the high-mannose and complex types have binding affinity for aromatic amino acid residues (*10*). This unique binding affinity of *N*-glycans, which seems to be attributable to their hydrophobic regions which are similar to the interior of cyclodextrin cavity, and which implies a novel interaction between *N*-glycans and proteins, prompted us to examine whether free *N*-glycans affect protease digestion of proteins. This report shows that free *N*-glycans protect proteins from protease digestion through their binding affinities for the solvent-exposed aromatic amino acid residues of both substrate proteins and proteases.

MATERIALS AND METHODS

Materials—RNase A (type IIIA), LA (type III, Ca^{2+}-depleted), horse kidney metallothionein, GlcNAc-Asn, cytidine 2′:3′-cCMP, fluorescamine, *N*-acetyl-L-tyrosine, and *N*-acetyl-L-tryptophan were all purchased from Sigma. Trypsin and chymotrypsin, treated with *N*-tosyl-L-phenylalanyl chloromethyl ketone and *N*-tosyl-L-lysyl chloromethyl ketone, respectively, were also from Sigma. α-*N*-Benzoyl-L-arginine ethyl ester was a product of Nacalai Tesque. An *N*-linked high-mannose type glycan, Manα1-2Manα1-6 (Manα1-2Manα1-3) Manα1-6 (Manα1-2Manα1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-Asn (M9-Asn), was obtained from soybean agglutinin as described previously (*11*). A mixture of *N*-linked biantennary glycans, ±NeuAcα2-6Galβ1-4GlcNAcβ1-2manα1-6 (±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4 (±Fucα1-6) GlcNAc-Asn (CII-Asn), was prepared by repeated pronase digestion of human apotransferrin (Nacalai Tesque), followed by gel-filtration on a Sephadex G-25 column. Another mixture of *N*-linked triantennary glycans, ±NeuAcα2-3Galβ1-4GlcNAcβ1-4 (±NeuAcα2-6Galβ1-4GlcNAcβ1-2) Manα1-6 (±NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc-Asn

[1] This study was supported in part by a Grant-in-Aid for Scientific Research (11480169) from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. E-mail: simplife@biochem.osakafu-u.ac.jp

Abbreviations: CII-Asn, ±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6 (±NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4 (±Fucα1-6)GlcNAc-Asn; CIII-Asn, ±NeuAcα2-3Galβ1-4GlcNAcβ1-4 (±NeuAcα2-6Galβ1-4GlcNAcβ1-2)Manα1-6(±NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn; LA, bovine α-lactalbumin; M9-Asn, Manα1-2Manα1-6(Manα1-2Manα1-3)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-Asn; TEA-HCl, 50 mM triethanolamine hydrochloride buffer, pH 8.0.

(CIII-Asn), was prepared from bovine serum fetuin (Seikagaku Kogyo) as described for CII-Asn after the starting material had been treated with alkaline borohydride to remove *O*-glycans (*12*). *N*-Glycans devoid of asparagine residues were prepared by pepsin digestion of the glycoproteins followed by glycopeptidase A digestion of the resulting glycopeptides as described previously (*11*). Other chemicals used here were described in the preceding papers (*7*, *8*).

Trypsin and Chymotrypsin Digestions—RNase A, LA, and metallothionein (100, 40, and 100 μM, respectively) were digested with protease at 40°C at an enzyme/substrate molar ratio of 1:4 in 50 mM triethanolamine hydrochloride buffer (pH 8.0) (TEA-HCl) containing 0.1 M NaCl and 10 mM $CaCl_2$. Trypsin digestion of a synthetic substrate, α-*N*-benzoyl-L-arginine ethyl ester, was performed essentially as described previously (*13*): the substrate (0.94 mM) was digested with trypsin (1.6 μM), and change in A_{253} was measured at 25°C for 3 min.

Assay of RNase A Activity—An aliquot (20 μl) of the protease digests of RNase A was diluted 50-fold with TEA-HCl containing 1 mM phenylmethanesulfonyl fluoride, 0.1 M NaCl, and 10 mM $CaCl_2$. The solution was mixed with 0.21 M cytidine 2′:3′-cCMP in TEA-HCl (20 μl, 25°C) containing 0.1 M NaCl and 10 mM $CaCl_2$, and then change in A_{296} was measured at 25°C for 10 min (*14*).

Fluorescence Measurements—Fluorescence measurements were performed with a Shimadzu RF-1500 fluorescence spectrophotometer at 25°C. Fluorescamine reaction for the estimation of the free amino groups produced by protease digestion was carried out essentially as described by Udenfriend *et al.* (*15*). To the protease digests (0.82 ml) was added 2 mM fluorescamine in acetone (80 μl), and then fluorescence was measured with excitation at 390 nm and emission at 475 nm. Tyrosyl and tryptophyl fluorescence spectra of the proteases were recorded with excitation at 268 and 280 nm, respectively, as described previously (*10*). Fluorescence measurements of 1-anilino-8-naphthalenesulfonate binding were also described previously (*8*, *9*).

Size-Exclusion HPLC of the Protease Digestion Products of LA—After stopping the digestion with 1 mM phenylmethanesulfonyl fluoride, 20 μl of the digest was applied to a TSK-GEL G3000SW column (0.75 × 30 cm) (Tosoh), which was developed with 50 mM Tris-acetate buffer (pH 7.2) at a flow rate of 0.5 ml/min. The elution of protein was monitored by measuring A_{225}.

RESULTS

Effects of Free N-Glycans on the Protease Digestion of RNase A—Three of the six tyrosine residues of RNase A are known to be solvent-exposed (*16*, *17*) and quantitatively *O*-acetylated with *N*-acetylimidazole under native conditions (*16*). We recently revealed that free *N*-glycans of both the high-mannose and complex types have binding affinity for these exposed tyrosine residues (*10*). It is interesting, therefore, to see whether free *N*-glycans affect the protease digestion of RNase A.

Figure 1 shows the time course of activity decrease during trypsin digestion of RNase A in the presence of various *N*-glycans. The mixture of complex-type *N*-linked triantennary glycans, CIII-Asn, distinctly depressed the decrease of RNase A activity, while the *N*-linked biantennary glycans, CII-Asn, and the high-mannose type glycan, M9-Asn, were somewhat less effective. The increasing order of the protective effects of the *N*-glycans on the protease digestions appears to parallel that of their affinities for aromatic amino acid residues (*10*), suggesting that the binding affinities of

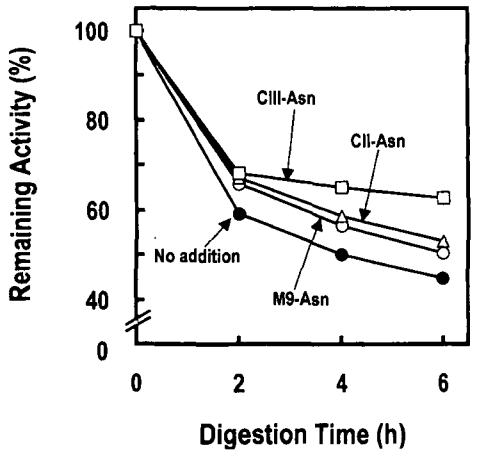


Fig. 1. **Effects of *N*-glycans on the trypsin digestion of RNase A.** RNase A was digested with trypsin in the presence or absence of 1 mM *N*-glycan, and the remaining activity was assayed. Each point represents the mean of at least three independent measurements differing by less than 9%.

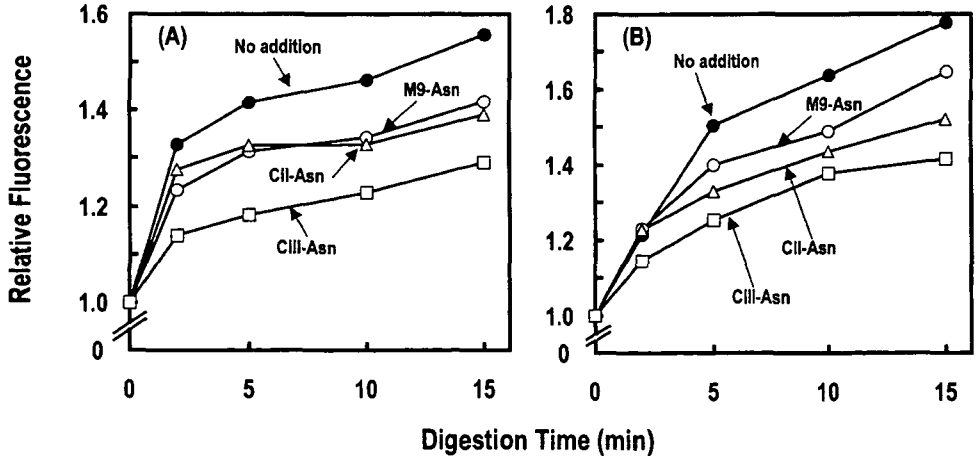


Fig. 2. **Effects of *N*-glycans on the trypsin (A) and chymotrypsin (B) digestions of RNase A.** RNase A was digested with a protease in the presence or absence of 1 mM *N*-glycan, and the amino group liberation was followed by fluorescamine labeling. Each point represents the mean of at least three independent measurements differing by less than 12%.

the *N*-glycans for the exposed tyrosine residues interfered with the proteolysis.

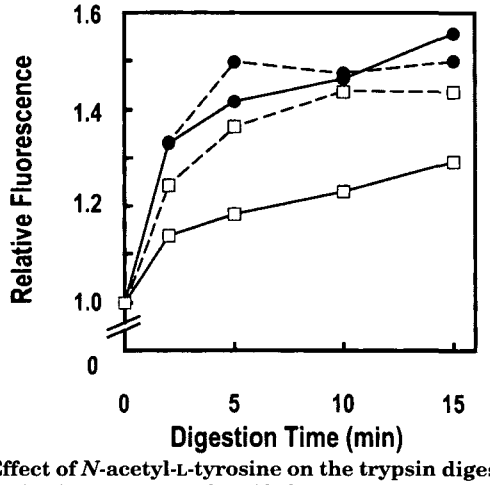


Fig. 3. **Effect of *N*-acetyl-L-tyrosine on the trypsin digestion of RNase A in the presence of an *N*-glycan.** RNase A was digested with trypsin in the presence (□) or absence (●) of 1 mM CIII-Asn, and in the presence (dashed lines) or absence (solid lines) of 50 mM *N*-acetyl-L-tyrosine. The protease digestion was followed by fluorescamine labeling of the liberated amino groups. Each point represents the mean of at least three independent measurements differing by less than 9%.

To gain more insight into the protective effect of the *N*-glycans on the protease digestion of RNase A, the time course of the digestion was examined by fluorescamine labeling of the newly liberated *N*-terminal amino groups.

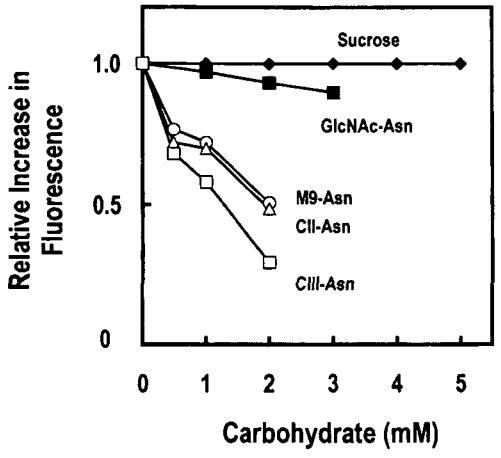
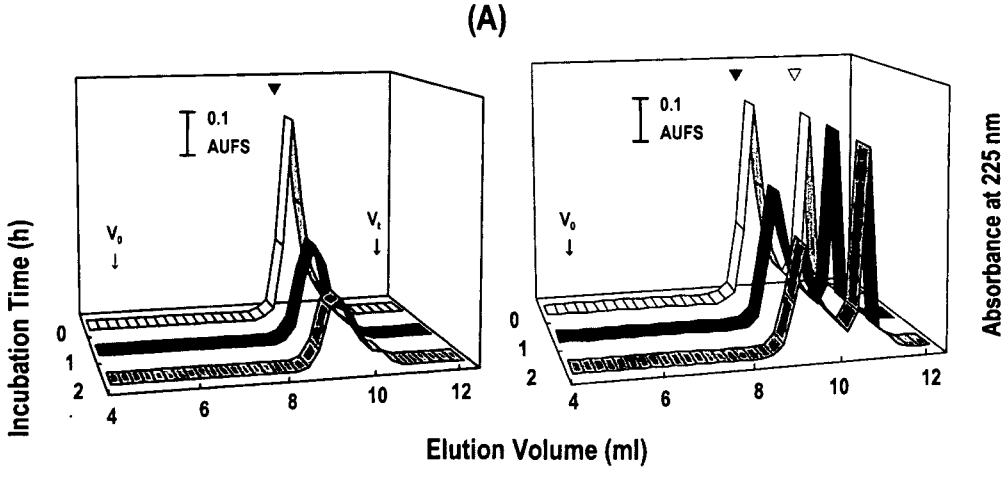


Fig. 4. **Dependence of the trypsin digestion of RNase A on carbohydrate concentration.** Trypsin digestion was performed with a carbohydrate at the indicated concentration, and the amino group liberation was followed by fluorescamine labeling. Each point represents the mean of at least two independent measurements.



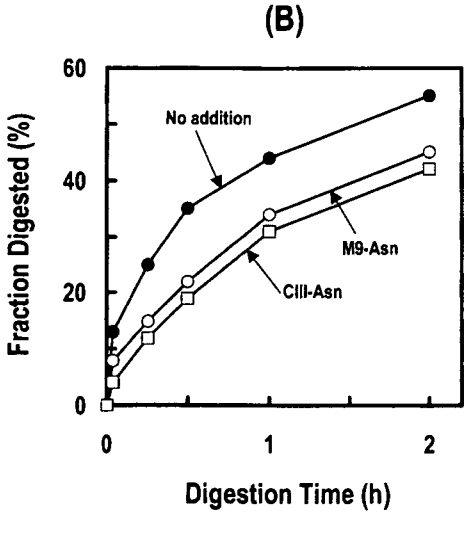


Fig. 5. **Effects of *N*-glycans on the trypsin digestion of LA.** (A) LA was digested with trypsin in the presence (right panel) or absence (left panel) of 1 mM M9-Asn. After stopping the digestion with 1 mM phenylmethanesulfonyl fluoride at the indicated times, the digests were submitted to size-exclusion HPLC. The filled and open arrowheads show the elution positions of the native LA and M9-Asn, respectively. (B) LA was digested with trypsin in the presence or absence of 1 mM *N*-glycan. After stopping the digestion with 1 mM phenylmethanesulfonyl fluoride at the indicated times, the digests were submitted to size-exclusion HPLC. Each point represents the mean of at least four independent measurements differing by less than 12%.

This highly sensitive fluorescent probe was expected to reveal the protective features of the *N*-glycans at an early stage of protease digestion. Contrary to the protective features reflected in the decrease in RNase A activity, the *N*-glycans were especially effective in the initial stages of the trypsin digestion of RNase A (Fig. 2A), and similar results were obtained with chymotrypsin digestion of RNase A (Fig. 2B). These results confirmed that the increasing order of the protective effects of the *N*-glycans parallels that of their affinities for aromatic groups. In this connection it is noteworthy that the presence of an aromatic amino acid derivative such as *N*-acetyl-L-tyrosine or *N*-acetyl-L-tryptophan greatly diminished the protective effects of the *N*-glycans. Figure 3 shows the effect of *N*-acetyl-L-tyrosine on the trypsin digestion of RNase A in the presence of CIII-Asn. *N*-Acetyl-L-tryptophan showed an even stronger effect and completely abolished the protective effects of the *N*-glycans on the trypsin digestion of RNase A (data not shown). These results firmly supported the view that the *N*-glycans sterically disturb the protease digestion by interacting with the aromatic amino acid residues exposed on the protein molecules.

As shown in Fig. 4, the effects of these *N*-glycans increased with their concentrations. The smallest *N*-glycan, GlcNAc-Asn, seemed to be slightly effective, whereas sucrose exhibited no detectable effect. The *N*-glycans devoid of asparagine residues were also effective, similarly to the "normal" *N*-glycans (data not shown).

Effects of N-Glycans on the Protease Digestion of LA—LA has two solvent-exposed tryptophan residues, which are quantitatively oxidized by *N*-bromosuccinimide (*18*). First, the effects of the *N*-glycans on the trypsin digestion of LA were examined by means of size-exclusion HPLC. Figure 5A shows the elution profiles of LA digested with trypsin in the presence or absence of 1 mM M9-Asn, which include only one protein peak at the position corresponding to the native LA. Peaks arising from partial proteolysis products and from trypsin were not detected. It seems likely that they were retained on the HPLC column and/or sample filters, as was the case with the refolding intermediates of LA (*9*). More detailed time courses of trypsin digestion of LA in the presence of *N*-glycans are shown in Fig. 5B. Next, the trypsin digestion of LA in the presence of *N*-glycans was followed by fluorescamine labeling (Fig. 6A). The *N*-glycans seemed to be especially effective at the initial stage of the digestion, and CIII-Asn, was more effective than M9-Asn, in agreement with the increasing order of their affinities for tryptophan residue (*10*). Similar results were obtained with chymotrypsin digestion of LA (Fig. 6B). The protective effects of the *N*-glycans on LA were practically abolished in the presence of *N*-acetyl-L-tryptophan (Fig. 7), as observed with RNase A. Further, the protective effects of the *N*-glycans increased with their concentrations, and GlcNAc-Asn and sucrose had no significant effects (Fig. 8). Thus there seems no doubt that *N*-glycans protect LA from protease digestion through their binding affinities for the solvent-exposed tryptophan residues.

Comparison of the Protective Effects of N-Glycans with Those of Common Carbohydrates with Different Structures —To determine whether the depressive effect on protease digestion is characteristic of *N*-glycans, the effects of some common carbohydrates on the proteolysis of RNase A and LA were examined. As summarized in Fig. 9, the com-

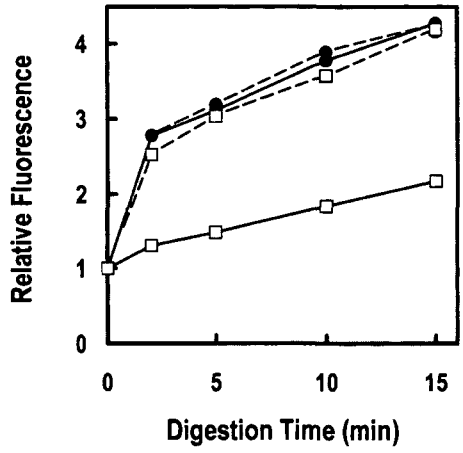


Fig. 7. **Effect of *N*-acetyl-L-tryptophan on the trypsin digestion of LA in the presence of an *N*-glycan.** LA was digested with trypsin in the presence (□) or absence (●) of 1 mM CIII-Asn, and in the presence (dashed lines) or absence (solid lines) of 50 mM *N*-acetyl-L-tryptophan. The protease digestion was followed by fluorescamine labeling of the liberated amino groups. Each point represents the mean of at least three independent measurements differing by less than 8%.

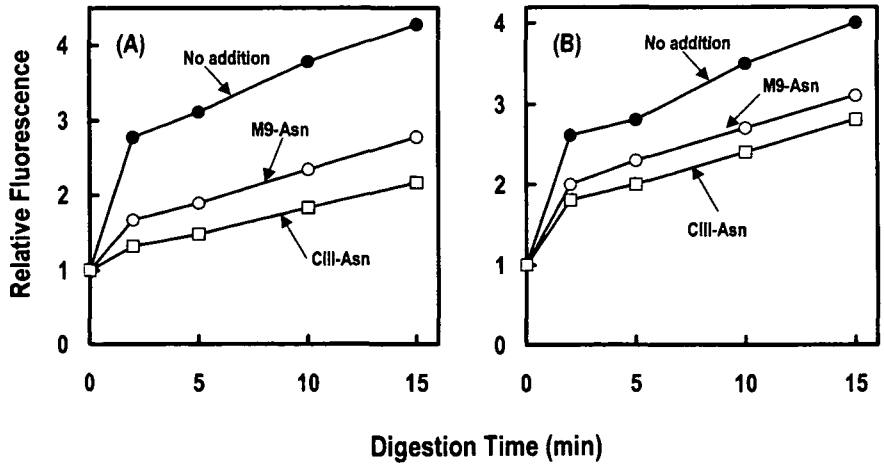


Fig. 6. **Effects of *N*-glycans on the trypsin (A) and chymotrypsin (B) digestion of LA.** LA was digested with a protease in the presence or absence of 1 mM *N*-glycan, and the amino group liberation was followed by fluorescamine labeling. Each point represents the mean of at least three independent measurements differing by less than 10%.

mon carbohydrates, but not yeast mannan, a high-mannose type *N*-glycan, had no significant effects on the protease digestions. These results, together with the above observations that the increasing order of the protective effects of the *N*-glycans parallels that of their affinities for aromatic amino acid residues and that the protective effects of the *N*-glycans are abolished by the presence of an aromatic amino acid, strongly suggest that the *N*-glycans protect proteins through their binding affinities for aromatic groups that are attributable to their glycan structures.

Effects of N-Glycans on Protease Activities—It is essential for a full understanding of the protective effect of *N*-glycans to reveal the interaction between *N*-glycans and proteases. First, we inquired whether trypsin and chymotrypsin have aromatic amino acid residues exposed on the protein molecules. As previously revealed, free *N*-glycans of both the high-mannose and complex types depress the intrinsic fluorescence intensities of proteins by interacting with the solvent-exposed aromatic amino acid residues (*10*). The tyrosyl and tryptophyl fluorescence intensities of both trypsin and chymotrypsin were markedly depressed in 1 mM solutions of *N*-glycans, as observed with RNase A and LA (*10*), showing that they have solvent-exposed aromatic amino acid residues (Fig. 10) (data shown only for trypsin). Nevertheless, no *N*-glycans exhibited detectable effects on the trypsin and chymotrypsin activities toward a low molecular-weight substrate, α-*N*-benzoyl-L-arginine ethyl ester (data not shown). On the other hand, the protease digestion of metallothionein, which is considered to have no significant affinity for *N*-glycans because of the lack of aromatic amino acid residues and also because its 1-anilino-8-naphthalenesulfonate binding is independent of the presence of free *N*-glycans (data not shown), was considerably depressed by the presence of *N*-glycans (Fig. 11) (data shown only for trypsin digestion). Because the increasing order of the

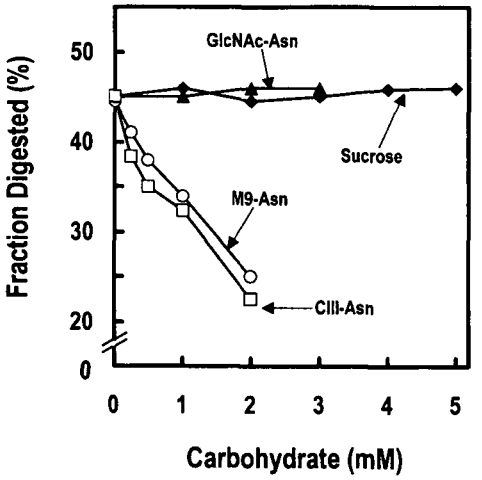


Fig. 8. **The dependence of the trypsin digestion of LA on the carbohydrate concentrations.** Trypsin digestion was performed in the presence of a carbohydrate at the indicated concentration, and the amino group liberation was followed by fluorescamine labeling. Each point represents the mean of at least two independent measurements.

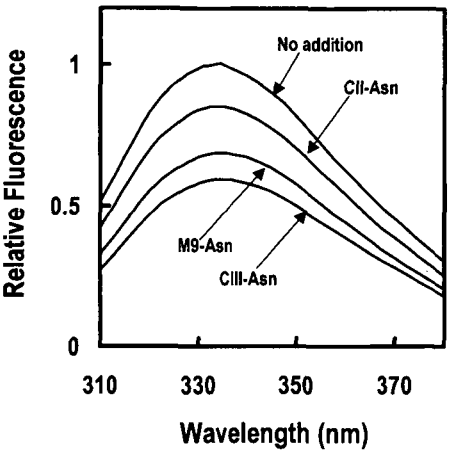


Fig. 10. **Effects of *N*-glycans on the intrinsic fluorescence of trypsin.** Spectra of the tryptophyl fluorescence of trypsin (2 μM) were recorded after incubation for 10 min in the presence or absence of 1 mM *N*-glycan in 50 mM Tris-HCl (pH 7.8) containing 0.1 M NaCl and 10 mM $CaCl_2$. The data are representative of two similar experiments.

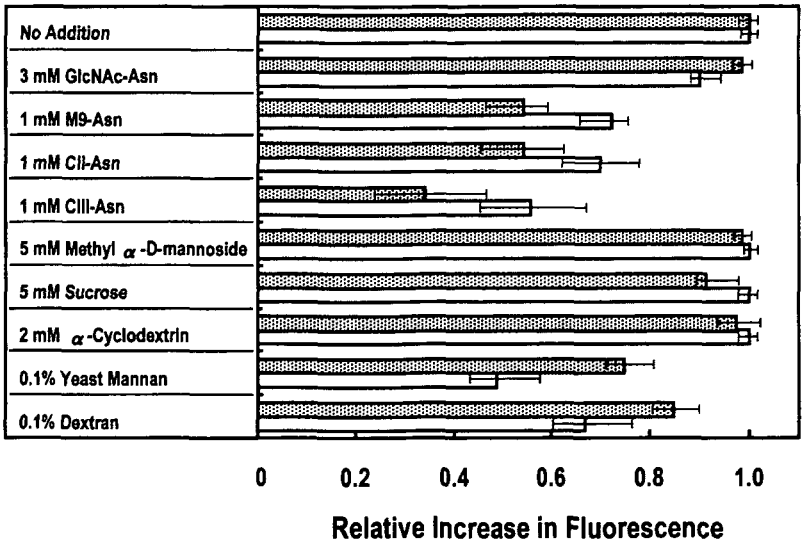


Fig. 9. **Depressive effects of various carbohydrates on the trypsin digestion of RNase A and LA.** RNase A (open bars) and LA (shaded bars) were digested with trypsin for 15 min with a carbohydrate at the indicated concentration, then the liberated amino groups were estimated by fluorescamine labeling. The data represent at least three independent measurements and are expressed as relative increase in fluorescence, where 1 corresponds to the increase of the *N*-terminal amino groups of RNase A and LA at 15 min after the start of protease digestion in the absence of a carbohydrate. Error bars show the maximum and minimum values of the repeated measurements.

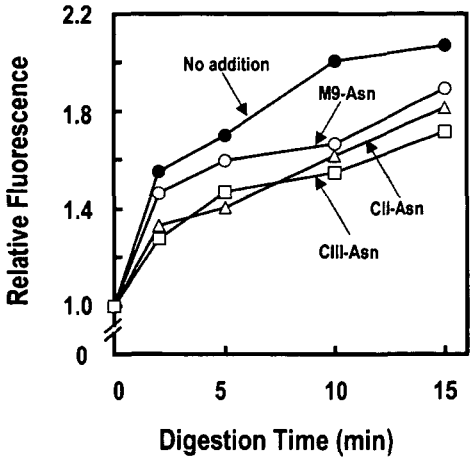


Fig. 11. **Effects of *N*-glycans on the trypsin digestion of metallothionein.** Metallothionein was digested with trypsin in the presence or absence of 1 mM *N*-glycan, and the amino group liberation was followed by fluorescamine labeling. Each point represents the mean of at least three independent measurements differing by less than 14%.

protective effects of the *N*-glycans on the protease digestion of metallothionein parallels that of their affinities for aromatic amino acid residues, the interaction of the *N*-glycans with the aromatic amino acid residues exposed on the protease molecules seems to have sterically hindered the enzymatic actions on metallothionein.

DISCUSSION

The most probable conclusion from the evidence at hand is that the *N*-glycans protect proteins from protease digestion through their binding affinities for the solvent-exposed aromatic amino acid residues. Most proteins, including proteases, have a number of solvent-exposed aromatic amino acid residues; *i.e.*, free *N*-glycans should usually have affinity for proteases as well as for substrate proteins. To examine the direct influence of free *N*-glycans on protease activity, therefore, it is desirable to use a protein substrate with no significant binding affinity for *N*-glycans. For this purpose, metallothionein, which is one of the few aromatic amino acid–free proteins readily available in sufficient quantity, seemed to be an apposite protein substrate. The findings that the *N*-glycans depress the protease digestion of metallothionein, whereas they do not affect the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester, show that the protease digestion of proteins could be sterically hindered by the binding of the *N*-glycans with the aromatic amino acid residues exposed on protease molecules. Thus it is almost certain that the *N*-glycans protect proteins from protease digestion through their binding affinities for the solvent-exposed aromatic amino acid residues of both substrate proteins and proteases. It is still uncertain, however, whether *N*-glycans have binding affinity for the aliphatic side chains of hydrophobic amino acids such as isoleucine, leucine, and proline.

The *N*-glycans appear to exhibit their protective effects especially in early stages of the protease digestion, suggesting that the initial hydrolysis of peptide linkages turns the rigid protein molecules into rather flexible ones that are more readily accessible to protease digestion. In this study, a high level of proteases was conveniently used to quickly follow the protease digestion. With a relatively low level of proteases, therefore, *N*-glycans could be expected to be much more effective in protecting proteins from digestion.

It has recently become apparent that free oligosaccharides of both the high-mannose and complex types are contained in endoplasmic reticulum (*19*), cytosol (*20–22*), fish eggs (*23*, *24*), and plant cells (*25–27*) in substantial amounts. The physiological significance of these oligosaccharides has so far been studied primarily in relation to the degradation pathway of glycoproteins (*20*, *28*, *29*). However, the functional importance of the oligosaccharides is suggested by the fact that free polymannose oligosaccharides are released from the endoplasmic reticulum immediately after their *N*-glycosylation there and translocated to the lysosome *via* the cytosol by a circuitous route (*19*). Actually, it is known that free oligosaccharides of both the high-mannose and complex types directly promote protein folding *in vitro* (*7–9*), presumably through their affinity for the aromatic amino acid residues (*10*). It appears, therefore, that particular attention should be paid to the potential *in vivo* functions of the extramolecular free oligosaccharides, as in the case of the intramolecular *N*-glycans of glycoproteins. In view of the results and arguments presented here, it would not be surprising if these oligosaccharides were involved in such physiological functions as stabilization of proteins and regulation of physiological activities.

REFERENCES

1. Grafl, R., Lang, K., Vogl, H., and Schmidt, F.X. (1987) The mechanism of folding of pancreatic ribonuclease is independent of the presence of covalently linked carbohydrate. *J. Biol. Chem.* **262**, 10624–10629
2. Narhi, L.O., Arakawa, T., Aoki, K.H., Elmore, R., Rohde, M.F., Boone, T., and Strickland, T.W. (1991) The effect of carbohydrate on the structure and stability of erythropoietin. *J. Biol. Chem.* **266**, 23022–23026
3. Wyss, D.F., Withka, J.M., Knoppers, M.H., Sterne, K.A., Recny, M.A., and Wagner, G. (1993) ^{1}H resonance assignments and secondary structure of the 13.6 kDa glycosylated adhesion domain of human CD2. *Biochemistry* **32**, 10995–11006
4. Meldgaard, M. and Svendsen, I. (1994) Different effects of *N*-glycosylation on the thermostability of highly homologous bacterial (1,3-1,4)-β-glucanases secreted from yeast. *Microbiology* **140**, 159–166
5. Wyss, D.F., Choi, J.S., Li, J., Knoppers, M.H., Willis, K.J., Arulanandam, A.R.N., Smolyar, A., Reinherz, E.L., and Wagner, G. (1995) Conformation and function of the *N*-linked glycan in the adhesion domain of human CD2. *Science* **269**, 1273–1278
6. Wang, C., Eufemi, M., Turano, C., and Giartosio, A. (1996) Influence of the carbohydrate moiety on the stability of glycoproteins. *Biochemistry* **35**, 7299–7307
7. Yamaguchi, H. and Uchida, M. (1996) A chaperone-like function of intramolecular high-mannose type chains in the oxidative refolding of bovine pancreatic RNase B. *J. Biochem.* **120**, 474–477
8. Nishimura, I., Uchida, M., Inohana, Y., Setoh, K., Daba, K., Nishimura, S., and Yamaguchi, H. (1998) Oxidative refolding of bovine pancreatic RNases A and B promoted by Asn-glycans. *J. Biochem.* **123**, 516–520
9. Kimura, N., Uchida, M., Nishimura, S., and Yamaguchi, H. (1998) Promotion of polypeptide folding by interactions with Asn-glycans. *J. Biochem.* **124**, 857–862
10. Yamaguchi, H., Nishiyama, T., and Uchida, M. (1999) Binding affinity of *N*-glycans for aromatic amino acid residues: Implications for novel interactions between *N*-glycans and proteins. *J.*

Biochem. **126**, 261–265
11. Nagai, K., Shibata, K., and Yamaguchi, H. (1993) Role of intramolecular high-mannose chains in the folding and assembly of soybean (*Glycine max*) lectin polypeptides: Studies by the combined use of spectroscopy and gel-filtration size-analysis. *J. Biochem.* **114**, 830–834
12. Spiro, R.G. and Bhoyroo, V.D. (1974) Structure of the *O*-glycosidically linked carbohydrate units of fetuin. *J. Biol. Chem.* **249**, 5704– 5717
13. Schwert, G.W. and Takenaka, Y. (1955) A spectrophotometric determination of trypsin and chymotrypsin. *Biochim. Biophys. Acta* **16**, 570–575
14. Crook, E.M., Mathias, A.P., and Rabin, B.R. (1960) Spectrophotometric assay of bovine pancreatic ribonuclease by the use of cytidine 2′:3′-phosphate. *Biochem. J.* **74**, 234–238
15. Udenfriend, S., Stein, S., Bohlen, P., Leimgruber, W., and Weigele, M. (1972) Fluorescamine: A reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* **178**, 871–872
16. Simpson, R.T. and Vallee, B.L. (1966) Side-chain cotton effects of ribonuclease. *Biochemistry* **5**, 2531–2538
17. Egan, W., Shindo, H., and Cohen, J.S. (1978) On the tyrosine residues of ribonuclease A. *J. Biol. Chem.* **253**, 16–17
18. Bell, J.E., Castellino, F.J., Trayer, I.P., and Hill, R.L. (1975) Modification of bovine α-lactalbumin with *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzylbromide. *J. Biol. Chem.* **250**, 7579–7585
19. Weng, S. and Spiro, R. (1997) Demonstration of a peptide: *N*-glycosidase in the endoplasmic reticulum. *Biochem. J.* **322**, 655–661
20. Cacan, R., Dengremont, C., Labiau, O., Kmiecik, D., Mir, A.-M., and Verbert, A. (1996) Occurrence of a cytosolic neutral chitobiase activity involved in oligomannoside degradation: a study with Madin Darby bovine kidney (MDBK) cells. *Biochem. J.* **313**, 597–602
21. Iwai, K., Mega, T., and Hase, S. (1999) Detection of $Man_5GlcNAc$ and related free oligomannosides in the cytosol fraction of hen oviduct. *J. Biochem.* **125**, 70–74
22. Ohashi, S., Iwai, K., Mega, T., and Hase, S. (1999) Quantitation and isomeric structure analysis of free oligosaccharides present in the cytosol fraction of mouse liver: Detection of a free disialo biantennary oligosaccharide and glucosylated oligomannosides. *J. Biochem.* **126**, 852–858
23. Taguchi, T., Kitajima, K., Muto, Y., Inoue, S., Khoo, K.H., Morris, H.R., Dell, A., Wallace, R.A., Selman, K., and Inoue, Y. (1995) A precise structural analysis of a fertilization-associated carbohydrate-rich glycopeptide isolated from the fertilized eggs of euryhaline killi fish (*Fundulus heteroclitus*). Novel pentaantennary *N*-glycan chains with a bisecting *N*-acetylglucosaminyl residue. *Glycobiology* **5**, 611–624
24. Taguchi, T., Iwasaki, M., Muto, Y., Kitajima, K., Inoue, S., Khoo, K.H., Morris, H.R., Dell, A., and Inoue, Y. (1996) Occurrence and structural analysis of highly sulfated multiantennary *N*-linked glycan chains derived from a fertilization-associated carbohydrate rich glycoprotein in unfertilized eggs of *Tribolodon hakonensis*. *Eur. J. Biochem.* **238**, 357–367
25. Priem, B., Gitti, R., Bush, C.A., and Gross, K.C. (1993) Arabinose is a constituent of a plant *N*-glycan. *Plant Physiol.* **102**, 445–458
26. Kimura, Y., Takagi, S., and Shiraishi, T. (1997) Occurrence of free *N*-glycans in pea (*Pisum sativum* L) seedlings. Biosci. *Biotechnol. Biochem.* **61**, 924–926
27. Kimura, Y. (1999) Structural feature of free *N*-glycans in plant cells and substrate specificity of plant endoglycosidase. *Glycoconjugate J.* **16**, S97
28. Moore, S.E.H. and Spiro, R.G. (1994) Intracellular compartmentalization and degradation of free polymannose oligosaccharides released during glycoprotein biosynthesis. *J. Biol. Chem.* **269**, 12715–12721
29. Saint-pol, A., Bauvy, C., Codogno, P., and Moore, S.E. (1997) Transfer of free polymannosetype oligosaccharides from the cytosol to lysosome in cultured human hepatocellular carcinoma HepG2 cells. *J. Cell Biol.* **136**, 45–59