Biochemical and Biophysical Research Communications **267,** 134–138 (2000)
doi:10.1006/bbrc.1999.1963, available online at http://www.idealibrary.com on IDEAL®

Synthesis of Neoglycoenzymes with Homogeneous N-Linked Oligosaccharides Using Immobilized Endo-β-*N*-acetylglucosaminidase A

Kiyotaka Fujita,* Naotaka Tanaka,* Mutsumi Sano,† Ikunoshin Kato,†
Yasuhiko Asada,* and Kaoru Takegawa*,[1]

**Department of Life Sciences, Faculty of Agriculture, Kagawa University, Miki-cho, Kagawa 761-0795, Japan; and*
†Biotechnology Research Laboratory, Takara Shuzo Co. Ltd., Otsu, Shiga 520-2193, Japan

Received November 22, 1999

A procedure for the enzymatic synthesis of neoglycoenzymes is described. The gene encoding endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) was overexpressed in *Escherichia coli* as a fusion protein linked to glutathione *S*-transferase (GST). GST–Endo-A fusion was extracted as a soluble protein. The fusion protein was purified to homogeneity with glutathione–Sepharose 4B and showed transglycosylation activity toward high-mannose-type glycopeptides without removing the GST moiety. The GST–Endo-A immobilized on glutathione–Sepharose 4B retained its transglycosylation activity. The immobilized enzyme could transfer $(Man)_6GlcNAc$ *en bloc* to partially deglycosylated ribonuclease B without damaging its enzyme activity. The immobilized GST–Endo-A should be very useful for synthesizing active neoglycoenzymes attached with homogeneous N-linked oligosaccharides.

Endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96) hydrolyzes the *N,N′*-diacetylchitobiose moiety of asparagine-linked oligosaccharides of various glycoproteins. This enzyme is useful for the structural analysis of glycoproteins and can be used to isolate both N-linked oligosaccharides and partially deglycosylated proteins without damaging them. Endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) shows a strong transglycosylation activity unlike endo-β-*N*-acetylglucosaminidases, and oligosaccharides are transferred to a suitable acceptor such as GlcNAc, gentiobiose, and *p*-nitrophenyl-glucose during chitobiose core cleavage by the enzyme (1–4). Endo-A is a potential tool for remodeling of glycopeptides and glycoprotein; oligosaccharide can be transferred to GlcNAc-containing glycopeptides (5, 6). Moreover, using chemically synthesized glycopeptides containing GlcNAc or glucose as acceptor substrates, neoglycopeptides were enzymatically synthesized by Endo-A (6, 7).

The Endo-A gene was cloned, and its nucleotide sequence was determined (8). The cloned gene was expressed in *E. coli* by its own promoter, and the properties of the recombinant Endo-A were very similar to those of the native Endo-A purified from *A. protophormiae*. However, the production of Endo-A by its own promoter was not enough in *E. coli* cells, and the procedure for the preparation of purified enzyme was laborious. Therefore, a simple method for obtaining large amounts of purified Endo-A is required.

The reaction course of Endo-A in aqueous solution was examined (2, 9). The transglycosylation product was formed predominantly in the initial stage of the reaction. As the reaction proceeded, the product was rapidly used by the same enzyme. Since the transglycosylation product is a substrate for the same enzyme, the product is degraded and the process will repeat itself until all substrate is transferred to water. Therefore, the enzyme reaction needs to be stopped quickly to obtain a lot of transglycosylation product. We have used boiling treatment and protein denaturant to terminate the reaction. However, these procedures lead to denaturation of the protein moiety and cannot be used for the synthesis of enzymatically active glycoproteins.

To overcome these problems, we attempted to overexpress Endo-A as a fusion protein linked to GST. Interestingly, we found that not only GST–Endo-A fusion protein but also the immobilized GST–Endo-A retained the transglycosylation activity. Taking advantage of this finding, we describe a new method for the enzymatic synthesis of active neoglycoenzymes attached to homogeneous oligosaccharides by immobilized GST–Endo-A fusion protein.

[1] To whom correspondence should be addressed. Fax: +81-87-891-3021. E-mail: takegawa@ag.kagawa-u.ac.jp.

0006-291X/00 $35.00

MATERIALS AND METHODS

Materials. DNA restriction and modifying enzymes were from Takara Shuzo Co. (Kyoto, Japan). Bovine pancreatic ribonuclease B (RNase B) was purchased from Sigma (Type III-B) and further purified by concanavalin A (Con A)-agarose column chromatography. Glutathione-Sepharose 4B and pGEX-2T were from Amersham Pharmacia Biotech. Microcentrifuge membrane filter (Ultrafree C3 GV, pore size 0.22 μm) was from Millipore. All other chemicals were purchased from Wako Pure Chemicals Co. (Osaka, Japan).

Construction of expression plasmid and expression of GST–Endo-A fusion protein. The Endo-A gene was amplified by PCR from a pBluescript-derived clone (pKY1) as described previously (8). A forward primer was designed to position a *Bam*HI and a Factor Xa cleavage sites on the 5′-end at the junction of the signal peptide and the amino-terminus of Endo-A, 5′-GCGTGGATCCATCGAAGGTC-GATCTACGTACAACGGC-3′, and a reverse primer was designed to position the 3′-end beyond the termination codon, 5′-TCCGTG-TCTGGATCCTAAAAC-3′. The amplified fragment was digested with *Bam*HI, and the resultant restriction fragment was cloned into the *Bam*HI site of the GST fusion vector pGEX-2T. The pGEX-2T/Endo-A plasmid was transformed into *E. coli* JM109.

The *E. coli* cells containing pGEX-2T/Endo-A plasmid were grown at 37°C until the absorbance at 600 nm reached 0.4, and then exposed to 0.2 mM (final concentration) IPTG at 37°C for 2 h. The cells were collected by centrifugation and the cell pellet was resuspended in 40 ml of lysis buffer (50 mM Tris–chloride and 1 mM EDTA, pH 8.0), and then stored at −80°C.

Affinity purification of GST–Endo-A fusion protein. All operations in the enzyme purification were carried out at 4°C. The frozen cells were suspended in phosphate-buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and were sonicated three times for 1 min. The mixture of lysed cells was centrifuged at 10,000*g* for 30 min. The supernatant was mixed with glutathione–Sepharose 4B (1 ml) equilibrated with PBS for 12 h at 4°C and put on a column. The column was washed 3 times with 10 bed volumes of PBS buffer. The fusion protein was eluted using 50 mM Tris–HCl (pH 8.0) containing 10 mM glutathione, and was dialyzed against 10 mM phosphate buffer (pH 7.0). The purified GST–Endo-A fusion protein was digested by Factor Xa (1% w/w) in 50 mM Tris–HCl buffer (pH 8.0) containing 100 mM NaCl and 1 mM $CaCl_2$ for 24 h at 22°C. Then the GST moiety was removed using a glutathione–Sepharose 4B column.

Endo-A assay and analysis of transglycosylation products. Endo-β-*N*-acetylglucosaminidase activity was assayed with $(Man)_6(GlcNAc)_2$Asn-dansyl as the substrate (10). One unit was defined as the amount yielding 1 μmol of GlcNAc-Asn-dansyl per minute at 37°C under the assay conditions. $(Man)_6(GlcNAc)_2$Asn was prepared from ovalbumin glycopeptides by the method of Huang *et al.* (11). The transglycosylation of $(Man)_6(GlcNAc)_2$Asn to glucose by Endo-A was done as follows: 1.3 mg of $(Man)_6(GlcNAc)_2$Asn was incubated with 10 munits of Endo-A in 0.27 ml of 50 mM ammonium acetate buffer (pH 6.0) in the presence of glucose (final concentration, 0.1 M). After incubation of the mixture for 10 min at 37°C, the reaction was stopped by boiling the reaction mixture for 3 min. The reaction mixture was applied to a Superdex peptide HR10/30 column (10 × 300 mm) (Amersham Pharmacia Biotech) and eluted with water to remove free glucose. Thin-layer chromatography (TLC) was performed on silica gel 60 plates (Merck Art. 5626). For the separation and purification of the oligosaccharides, the following solvent system was used: *n*-propanol/acetic acid/water (v/v) = 3/3/2. The orcinol–H2SO4 reagent was used for the detection of the oligosaccharides (12).

Preparation of partially deglycosylated RNase B. The RNase B was partially deglycosylated by endo-β-*N*-acetylglucosaminidase from *Flavobacterium* sp., and GlcNAc-RNase was prepared as described previously (5, 13). After dialysis against 10 mM acetate buffer (pH 6.0), GlcNAc-RNase was placed on a column (1.5 × 6 cm) containing Con A-agarose at 4°C. GlcNAc-RNase was recovered from unbound fractions with the Con A column, and was dialyzed with distilled water.

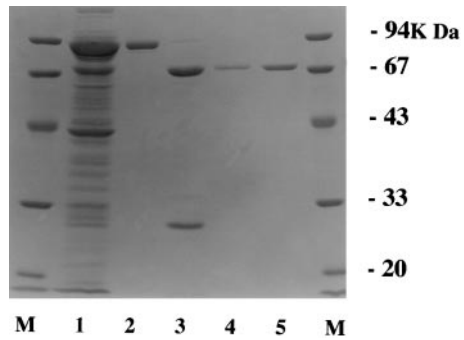

FIG. 1. SDS–PAGE analysis of GST–Endo-A fusion protein. The samples were loaded onto a 10% gel. Lane M, molecular weight markers; lane 1, soluble extract obtained from sonicated cells; lane 2, GST–Endo-A fusion purified by glutathione–Sepharose 4B affinity chromatography; lane 3, the fusion protein was cleaved by Factor Xa; lane 4, lane 3 was further purified on a glutathione–Sepharose 4B column; lane 5, native Endo-A purified from *A. protohpormiae.*

Analytical methods. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was done by the standard methods (14). The molecular weight markers were purchased from Amersham Pharmacia Biotech. Proteins were visualized with Coomassie brilliant blue R-250. Lectin blotting was done by the procedure of Ogawa *et al.* (15). The proteins were separated by SDS–PAGE, and blotting onto a polyvinylidene difluoride membrane. The membrane was stained with Con A conjugated to horseradish peroxidase (HRP). The glycoproteins were detected by using 3,3′-diaminobenzidine/H_2O_2 as substrate for HRP. The activity of RNase toward cytidine 2′,3′-cyclic monophosphate was monitored as the increase in optical density at 284 nm (16).

RESULTS AND DISCUSSION

Expression and purification of GST–Endo-A fusion protein. The Endo-A gene was inserted into an expression vector, and was expressed in *E. coli* as GST fusion protein following induction from the strong P (tac) promoter by IPTG. The fusion protein was recovered in the soluble fraction (Fig. 1, lane 1), and purified with a glutathione–Sepharose 4B column. The purified GST–Endo-A preparation showed a single protein band on SDS–PAGE corresponding to a molecular weight of 96 kDa (Fig. 1, lane 2). Following digestion with Factor Xa, the purified GST–Endo-A was converted into two bands with Endo-A (69 kDa) and GST (26 kDa) (Fig. 1, lane 3). The recombinant Endo-A was further purified using glutathione-Sepharose to remove free GST protein (Fig. 1, lane 4). Thus, we have obtained large amounts of purified recombinant Endo-A by a simple isolation method.

During the purification of recombinant Endo-A, we noticed that the GST–Endo-A fusion protein shows hydrolytic activity toward $(Man)_6(GlcNAc)_2$Asn-dansyl without removing the GST moiety. The enzymatic properties of GST–Endo-A were compared with those

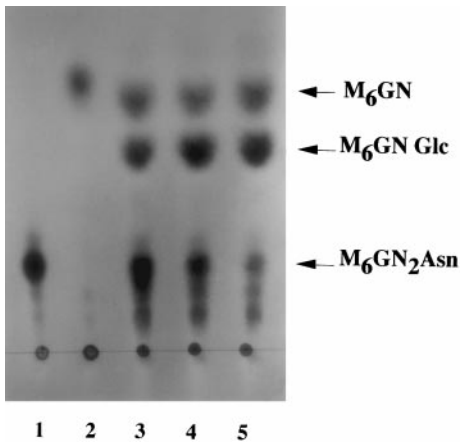

FIG. 2. TLC analysis of the transglycosylation products. $(Man)_6(GlcNAc)_2Asn$ was incubated with native Endo-A (lane 3) or GST–fusion Endo-A (lane 4) in the presence of 0.5 M glucose for 10 min at 37°C. The immobilized GST–Endo-A was used for transglycosylation (lane 5). Lane 1, $(Man)_6(GlcNAc)_2Asn$; lane 2, standard $Man_6GlcNAc$.

of the native Endo-A. The specific activity of the purified GST–Endo-A was 4.26 units/mg, and that of purified native Endo-A from *E. coli* extract was 4.92 units/mg. The two enzymes also showed similar pH–activity profiles (both showed a broad optimum pH–activity profile and were most active in the range of pH 5.0 to 9.0). Both enzymes were stable at up to 60°C after incubation for 10 min. These results showed that the addition of GST in the N-terminal region of Endo-A caused no changes in the pH–activity–stability profile or in the kinetic parameters of the hydrolysis of $(Man)_6(GlcNAc)_2Asn$.

The transglycosylation of $(Man)_6(GlcNAc)_2Asn$ to glucose by GST–Endo-A was examined as described under Materials and Methods. The transglycosylation products were analyzed by TLC. One oligosaccharide spot was newly generated in the presence of glucose (Fig. 2, lane 4). This spot was collected and the carbohydrate composition was determined to be Man:GlcNAc:Glc = 6:1.0:1.0 (relative to Man = 6). These results showed that the recombinant GST–Endo-A has equal transglycosylation activity to native Endo-A purified from *A. protophormiae.*

Preparation of immobilized GST–Endo-A. We attempted to use GST–Endo-A for immobilized enzyme binding to glutathione–Sepharose 4B. Purified GST–Endo-A was mixed with glutathione–Sepharose 4B for 12 h at 4°C in the presence of PBS buffer. The beads were put on a column and were washed with PBS buffer. The enzyme activity of the immobilized GST–Endo-A was 0.12 units/ml (50% v/v bead suspension in PBS). The stability of the immobilized GST–Endo-A was confirmed with the microcentrifuge membrane filter as shown in Fig. 3A. Fifty nanomoles of $(Man)_6(GlcNAc)_2Asn$-DNS was incubated with 10 μl of immobilized GST–Endo-A in the presence of 1× PBS buffer (total volume, 35 μl). After incubation for 10 min at 37°C, the reaction was stopped by the centrifugation of the filter at 5000*g* for 10 s. The immobilized enzyme maintained enzyme activity for over 10 cycles of this procedure (Fig. 3B), and leakage of enzyme from Sepharose beads was not detected under these conditions. These results suggest that the immobilized GST–Endo-A can stand repetitive use of the enzymatic reaction.

The transglycosylation of $(Man)_6(GlcNAc)_2Asn$ to glucose by the immobilized GST–Endo-A was induced as follows. One mg of $(Man)_6(GlcNAc)_2Asn$ was incubated with 10 munits of immobilized Endo-A in the presence of 1× PBS buffer, and 100 mM glucose (total volume, 40 μl) added. After incubation for 10 min at

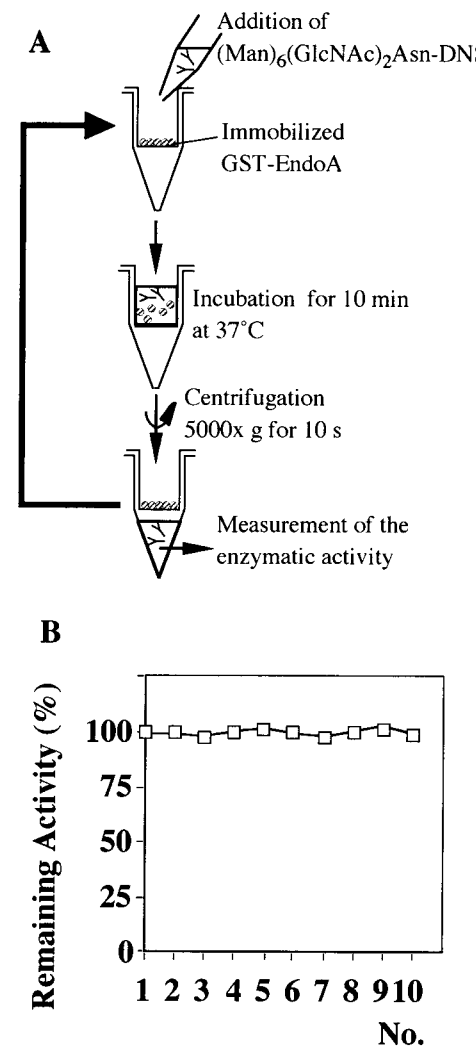

FIG. 3. Enzymatic reaction of the immobilized GST–Endo-A on the microcentrifuge membrane filter. (A) A scheme for the test of reactivity and stability of the immobilized enzyme. (B) Stability of the immobilized enzyme. These manipulations were repeated 10 times, and the remaining activity was determined by HPLC.

37°C, the reaction was stopped by centrifugation. The immobilized enzyme had similar transglycosylation activity to the native enzyme purified from *A. protophormiae* (Fig. 2, lane 5). It is expected that the immobilized GST–Endo-A will be useful for synthesizing neoglycoprotein without denaturation of the protein moiety.

Enzymatic synthesis of neo-RNase using immobilized GST–Endo-A fusion. We showed that oligosaccharide was transferred to partially deglycosylated RNase B by Endo-A. Our previous results indicate that the native Endo-A does not show oligosaccharide-transferring activity toward the native form of partially deglycosylated RNase B (5). We examined the transglycosylation to the partially deglycosylated RNase B without denaturation of protein moiety. The reaction conditions were as follows; 0.45 mg of $(Man)_6(GlcNAc)_2Asn$ was incubated with 10 munits of immobilized GST–Endo-A in the presence of 1× PBS buffer, then 10 mg of GlcNAc-RNase (total volume, 80 μl) added. After incubation for the indicated time at 37°C, the reaction mixture was analyzed by SDS–PAGE (Fig. 4). One protein band was newly generated with an increase of 2000 compared with the deglycosylated RNase B, and it migrated to the position of the native RNase B (4). The transglycosylation product peaked in content at 10 min, and was then gradually hydrolyzed by the same enzyme.

The reaction mixture was stained by lectin blotting using HRP-Con A (Fig. 5B). The transglycosylation product the same size as native RNase B was stained (Fig. 5B, lane 3), but GlcNAc-RNase was not (Fig. 5B, lane 2). The N-linked oligosaccharides of native RNase B consist of $(Man)_{6-9}(GlcNAc)$ (5). Neo-RNase was purified on a Con A–agarose column in the same way as RNase B, and the N-linked oligosaccharides of neo-RNase were confirmed to be modified to $(Man)_6(GlcNAc)_2$ analyzed by HPLC (5). We examined the enzymatic activity of neo-RNase in comparison with that of RNase B toward cytidine 2′,3′-cyclic monophosphate. They showed similar levels of activity and pH–activity profiles (data not shown), indicating that $(Man)_6GlcNAc$ was transferred to the deglycosylated RNase B without a change in enzymatic properties.

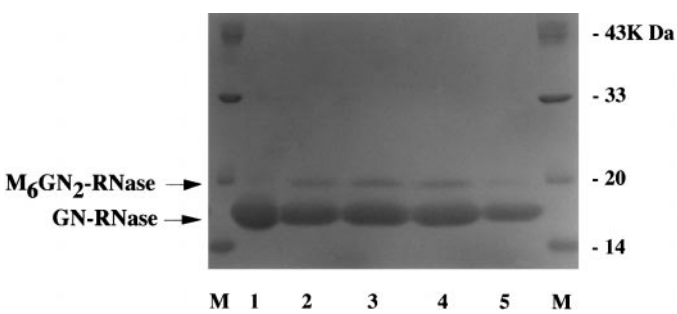

FIG. 4. Time course of enzymatic synthesis of Neo-RNase. $(Man)_6(GlcNAc)_2Asn$ was incubated with immobilized GST–Endo-A in the presence of GlcNAc-RNase for the indicated times at 37°C. The samples were loaded onto a 17% gel. Lane M; molecular weight markers, lane 1, 0 min; lane 2, 5 min; lane 3, 10 min; lane 4, 20 min; and lane 5, 40 min.

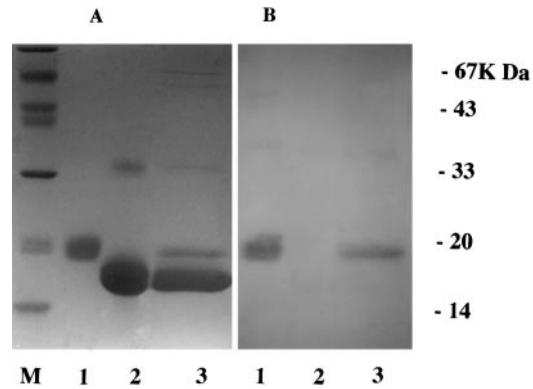

FIG. 5. Lectin blotting of neo-RNase. The samples were loaded onto a 15% gel. (A) Stained with Coomassie Brilliant Blue R-250. (B) Analyzed by lectin blotting using HRP–Con A. Lane M, molecular weight markers; lane 1, native RNase B; lane 2, GlcNAc-RNase B digested by endo-β-*N*-acetylglucosaminidase; lane 3, reaction mixture of neo-RNase.

It is difficult to study the biological roles of individual oligosaccharides in glycoproteins because naturally occurring glycoproteins have a high degree of heterogeneity in their oligosaccharide moiety. To our knowledge, this is the first report of the conversion of heterologous N-linked sugar chains to homogeneous sugar chains in active glycoenzymes. The method presented here is suitable for the synthesis of neoglycoenzymes without denaturation of protein moiety, and useful for attaching the same N-linked sugar chains to all the original glycosylation sites of glycoprotein molecules. Endo-A can readily deglycosylate RNase B but shows less oligosaccharide-transferring activity toward the native form of partially deglycosylated RNase B. Considering the steric effects of the protein moiety on the accessibility of glycosylation sites to Endo-A, steric factors may have a more direct influence upon the transglycosylation than upon hydrolytic activity. Efforts are underway to optimize the synthesis of neoglycoenzymes by the immobilized Endo-A fusion protein.

ACKNOWLEDGMENTS

We thank Akihiro Kondo (Takara Shuzo Co.) and Yuan C. Lee (Johns Hopkins University) for many helpful discussions during the course of this work. This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

1. Takegawa, K., Yamaguchi, S., Kondo, A., Iwamoto, H., Nakoshi, M., Kato, I., and Iwahara, S. (1991) *Biochem. Int.* **24,** 849–855.

2. Takegawa, K., Yamaguchi, S., Kondo, A., Kato, I., and Iwahara, S. (1991) *Biochem. Int.* **25,** 829–835.
3. Takegawa, K., Fujita, K., Fan, J. Q., Tabuchi, M., Tanaka, N., Kondo, A., Iwamoto, H., Kato, I., Lee, Y. C., and Iwahara, S. (1998) *Anal. Biochem.* **257,** 218–223.
4. Fan, J.-Q., Huynh, L. H., Reinhold, B. B., Reinhold, V. N., Takegawa, K., Iwahara, S., Kondo, A., Kato, I., and Lee, Y. C. (1996) *Glycoconj. J.* **13,** 643–652.
5. Takegawa, K., Tabuchi, M., Yamaguchi, S., Kondo, A., Kato, I., and Iwahara, S. (1995) *J. Biol. Chem.* **270,** 3094–3099.
6. Yamamoto, K., Haneda, K., Iguchi, R., Inazu, T., Mizuno, M., Takegawa, K., Kondo, A., and Kato, I. (1999) *J. Biosci. Bioeng.* **87,** 175–179.
7. Deras, I. L., Takegawa, K., Kondo, A., Kato, I., and Lee, Y. C. (1998) *Bioorg. Med. Chem. Lett.* **8,** 1763–1766.
8. Takegawa, K., Yamabe, K., Fujita, K., Tabuchi, M., Mita, M., Izu, H., Watanabe, A., Asada, Y., Sano, M., Kondo, A., Kato, I., and Iwahara, S. (1997) *Arch. Biochem. Biophys.* **338,** 22–28.
9. Fan, J.-Q., Takegawa, K., Iwahara, S., Kondo, A., Kato, I., Abeygunawardana, C., and Lee, Y. C. (1995) *J. Biol. Chem.* **270,** 17723–17729.
10. Takegawa, K., Nakoshi, M., Iwahara, S., Yamamoto, K., and Tochikura, T. (1989) *Appl. Environ. Microbiol.* **55,** 3107–3112.
11. Huang, C. C., Mayer, H. E., Jr., and Montgomery, R. (1970) *Carbohydr. Res.* **13,** 127–137.
12. Holmes, E. W., and O'Brien, J. S. (1979) *Anal. Biochem.* **93,** 167–170.
13. Yamamoto, K., Kadowaki, S., Takegawa, K., Kumagai, H., and Tochikura, T. (1986) *Agric. Biol. Chem.* **50,** 421–429.
14. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
15. Ogawa, H., Ueno, M., Uchibori, H., Matsumoto, I., and Seno, N. (1990) *Anal. Biochem.* **190,** 165–169.
16. Crook, E. M., Mathias, A. P., and Rabin B. R. (1960) *Biochem. J.* **74,** 234–238.