Chem. Pharm. Bull.
33(1) 276-281 (1985)

Kinetic and Circular Dichroism Spectroscopic Comparison among Three Forms of Glucoamylase from a *Rhizopus* sp.

TOMOKO TAKAHASHI,* MASANORI IWAMA, YUKIKO TSUCHIDA and MASACHIKA IRIE

Department of Microbiology, Hoshi College of Pharmacy, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142, Japan

(Received May 17, 1984)

Three forms of glucoamylase [EC 3.2.1.3] of a *Rhizopus* sp., $Gluc_1$ (M.W. 74000), $Gluc_2$ (M.W. 58600) and $Gluc_3$ (M.W. 61400), were compared by circular dichroism (CD) spectroscopy and kinetic studies. The CD spectra of the enzymes indicated that $Gluc_2$ and $Gluc_3$, which lack fragments from the N-terminal part of $Gluc_1$, resembled $Gluc_1$ in protein backbone conformation, although with some differences in the states of aromatic amino acid side chains. Therefore, the N-terminal part of $Gluc_1$ appears not to have a critical effect on the gross conformation of the remaining domain of $Gluc_1$, though two glycopeptides, presumably released from the N-terminal part of $Gluc_1$, fragments H (M.W. 16700) and L (M.W. (14400), had different conformations from that of $Gluc_1$. The three enzymes each contained 1 SH in a buried form, as well as 1 S-S bridge, and had similar susceptibility to denaturants. On the other hand, $Gluc_2$ and $Gluc_3$ differed markedly from $Gluc_1$ in the K_m values for large substrates, but differed little in the K_m and V_{max} values for small substrates or in the V_{max} values for large substrates, except for pullulan, which is highly branched. $Gluc_1$, but not $Gluc_2$ or $Gluc_3$, may have an additional site(s) interacting with large substrates, besides the active center.

Keywords——glucoamylase; *Rhizopus*; multiple forms; CD spectrum; denaturation; kinetic parameter; substrate-binding site

Three forms of glucoamylase [EC 3.2.1.3; α-D-(1→4)-glucan glucohydrolase] have been purified from a commercial digestive of a *Rhizopus* sp. and named $Gluc_1$ (M.W. 74000), $Gluc_2$ (M.W. 58600) and $Gluc_3$ (M.W. 61400) in order of content.[1] The three enzymes differed in both amino acid and sugar compositions but shared common antigenicity in immunodiffusion.[1,2] The N-terminal amino acids of the three enzymes were different, namely Ala for $Gluc_1$, Glu for $Gluc_2$ and Lys for $Gluc_3$, but their C-terminal amino acid sequences were the same: -Ser·Ala·OH.[2] In addition, two inactive glycopeptides, presumably released from the N-terminal region of $Gluc_1$, fragments H (M.W. 167000) and L (M.W. 14400), were successfully isolated from the commercial digestive.[2] It was therefore concluded that the two minor enzymes, $Gluc_2$ and $Gluc_3$, were derived from the most abundant enzyme, $Gluc_1$, by the action of a certain proteolytic enzyme(s) with concomitant liberation of N-terminal peptides of different sizes. Upon incubation with commercial proteinases such as papain and chymotrypsin, $Gluc_1$ was found to be convertible into $Gluc_2$-like and $Gluc_3$-like enzymes,[3] in strong support of the above conclusion.

It is of interest to see whether or not such removal of the N-terminal moiety of $Gluc_1$, resulting in the formation of $Gluc_2$ and $Gluc_3$, causes any change in the conformational and enzymatic properties. This line of study may lead to a better understanding of the structure-function correlations of glucoamylase. So far $Gluc_1$, $Gluc_2$ and $Gluc_3$ have been found to show close similarity in pH optimum, pH stability and heat stability, and to possess similar specific activities towards soluble starch in terms of units/mol enzyme.[1] During further comparison among the three enzymes, however, distinct differences were found in their kinetic

NII-Electronic Library Service

properties for high-molecular-weight substrates, including soluble starch.

In the present paper, we compare the conformations of $Gluc_1$, $Gluc_2$ and $Gluc_3$ as assessed by CD spectroscopy and their kinetic properties towards various substrates of different sizes.

Materials and Methods

Chemicals——Gluczyme (a commercial digestive from a *Rhizopus* sp.), used as a source of glucoamylase, was generously supplied by Amano Pharmaceutical Co. Soluble starch was purchased from Wako Pure Chemicals and used as a substrate after exhaustive dialysis against distilled water. Other substrates used were as follows: maltose and maltotriose (Wako Pure Chemicals), maltoheptaose (Boehringer Mannheim GmbH), amylose A (average degree of polymerization in glucose unit $\bar{n}=25$) (Nakarai Chemicals, Ltd.), amylopectin (Tokyo Kasei Kogyo Co., Ltd.), amylose EX-1 ($\bar{n}=17$) and pullulan (average M.W. 345000) (Hayashibara Biochem. Labs., Inc.), and *p*-nitrophenyl α-D-glucopyranoside (PNPG) and isomaltose (Koch-Light Lab. Ltd.). The D-glucose oxidase reagent (Glucose-Autotest), 5,5′-dithiobis(2-nitrobenzoic acid), urea and guanidine hydrochloride (Gu·HCl) were from Wako Pure Chemicals. All other chemicals were of analytical reagent grade.

Preparation of $Gluc_1$, $Gluc_2$ and $Gluc_3$ as Well as Fragments H and L——The three glucoamylases and the two fragments were purified from Gluczyme according to the methods[1,2] reported previously.

Estimation of Protein——Protein contents were estimated by the method of Lowry *et al.*[4] with bovine serum albumin as a standard. The concentrations of $Gluc_1$, $Gluc_2$ and $Gluc_3$ were estimated by using $A_{cm}^{280\,nm}$ (1%) values of 13.2, 13.7 and 13.4, respectively.[1]

Determination of Glucoamylase Activity——Glucoamylase activity was determined with soluble starch as a substrate according to the D-glucose oxidase method described previously.[5] One unit of glucoamylase activity was defined as the amount of enzyme liberating 1 μmol of glucose per min at 37 °C under the specified conditions.

Determination of Kinetic Parameters——Kinetic parameters, K_m and V_{max}, were measured as described previously[5] using soluble starch, glycogen, amylopectin and pullulan (0.03—0.2% for the former three and 0.1—2% for the last) as high-molecular-weight substrates, and maltose (1.0—7.0 mM), maltotriose (0.4—5.0 mM), maltoheptaose (0.2—2.0 mM), amylose EX-1 and amylose A (0.2—2.0 mM) PNPG (1.0—20 mM) and isomaltose (0.5—5.0 mM) as low-molecular-weight substrates; the values of K_m and V_{max} were estimated from Hofstee plots for the high-molecular-weight substrates and Lineweaver-Burk plots for the low-molecular-weight substrates.

Titration of SH Groups——Titration of free SH groups in protein was performed using 5,5′-dithiobis(2-nitrobenzoic acid) by the method of Ellman[6] as described previously.[5]

Measurement of CD Spectrum——CD spectra were measured at 25 °C with a JASCO J-40 spectropolarimeter in cells of 0.2-cm and 0.5-cm light path for the wavelength regions 200—250 and 250—320 nm, respectively; the protein concentrations used were 0.02% at 200—250 nm and 0.1% at 250—320 nm. All data are expressed as molar ellipticity ([θ])

Results

Conformations of $Gluc_1$, $Gluc_2$ and $Gluc_3$

The conformations of $Gluc_1$, $Gluc_2$ and $Gluc_3$ were examined by CD spectroscopy (Fig. 1). The CD spectra of the three enzymes in the shorter-wavelength region (200—250 nm), which are indicative of the backbone conformation of protein, were similar, although differing slightly in detail, and all showed a trough at around 218 nm with a shoulder at around 208 nm (Fig. 1a). On the other hand, the CD bands of $Gluc_2$ and $Gluc_3$ in the longer-wavelength region (250—320 nm), which are attributed to the aromatic side chains of amino acid residues in protein, were somewhat different from those of $Gluc_1$, though the peak (around 256, 263, 279—281 and 286 nm) and shoulder (around 250 and 270 nm) positions were still similar for the three enzymes (Fig. 1b). The CD spectra of fragments H and L, which correspond to N-terminal glycopeptide fragments of $Gluc_1$,[2] were also measured for comparison (Fig. 2). Both fragments H and L gave CD spectra different from those of the three enzymes not only in the shorter-wavelength region but also in the longer one; they showed a simple trough at 215 nm without any shoulder and a peak at 230 nm, followed by multi-peaks at 262—285 nm and a trough at around 297 nm. It was noted that the absolute values of [θ] for the fragments were much smaller (about one-tenth) than those for the three enzymes and that the N-terminal

NII-Electronic Library Service

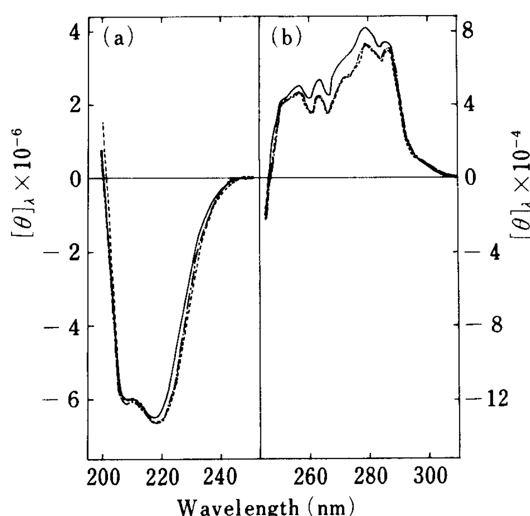

Fig. 1. CD Spectra of $Gluc_1$, $Gluc_2$ and $Gluc_3$

CD measurement was done for each enzyme in 0.01 M acetate buffer (pH 5.0) containing 0.1 M KCl. (a) CD spectra in the shorter-wavelength region. (b) CD spectra in the longer-wavelength region. ——, $Gluc_1$; ------, $Gluc_2$; — - —, $Gluc_3$.

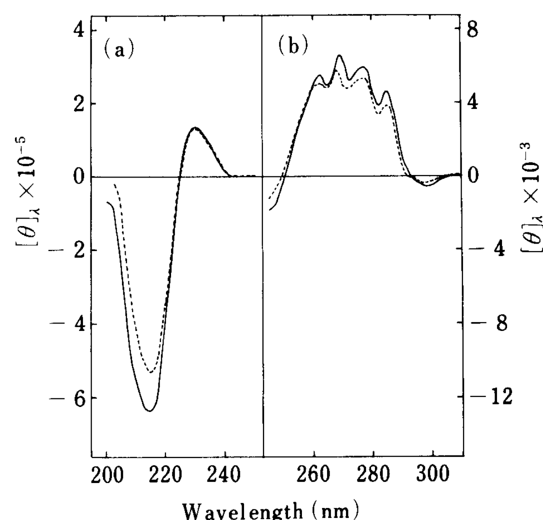

Fig. 2. CD Spectra of Fragments H and L

CD measurement was done under the same conditions as for Fig. 1. (a) CD spectra in the shorter-wavelength region. (b) CD spectra in the longer-wavelength region. ——, fragment H; ------, fragment L.

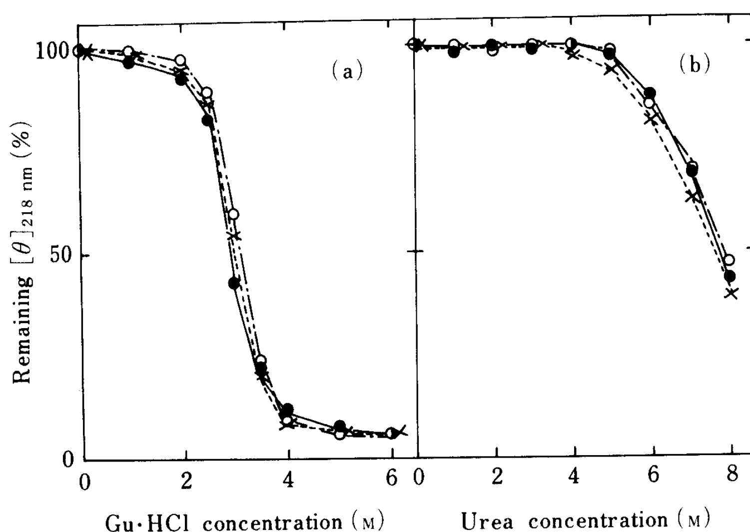

Fig. 3. Effects of Guanidine Hydrochloride and Urea on $Gluc_1$, $Gluc_2$ and $Gluc_3$

Each enzyme at 1 mg/ml of 0.01 M acetate buffer (pH 5.0) containing 0.1 M KCl was treated with the indicated concentrations of denaturant at room temperature for 2 h and then the CD spectra were measured. (a) The changes in $[\theta]_{218\,nm}$ of Gu·HCl-treated enzymes. (b) The changes in $[\theta]_{218\,nm}$ of urea-treated enzymes.
●—●, $Gluc_1$; ○- - -○, $Gluc_2$; ×---×, $Gluc_3$.

glycopeptide moiety of $Gluc_1$ appeared not to have a critical effect on the gross conformation of the remaining domain of the enzyme, as can be seen from Fig. 1.

Susceptibility of $Gluc_1$, $Gluc_2$ and $Gluc_3$ to Denaturants

To test whether or not loss of the N-terminal part of $Gluc_1$ causes any change in susceptibility to denaturants, the three enzymes were treated with various concentrations of Gu·HCl and urea. $Gluc_1$, $Gluc_2$ and $Gluc_3$ were found to have similar susceptibilities to the denaturants, as shown by the changes in $[\theta]_{218\,nm}$ (Fig. 3). Each enzyme started to be

NII-Electronic Library Service

TABLE I. Kinetic Parameters for $Gluc_1$, $Gluc_2$ and $Gluc_3$

Substrate	K_m			V_{max} (units × 10^{-2}/μmol)			$V_{max}/K_m \times 10^{-3}$		
	$Gluc_1$	$Gluc_2$	$Gluc_3$	$Gluc_1$	$Gluc_2$	$Gluc_3$	$Gluc_1$	$Gluc_2$	$Gluc_3$
Soluble starch	0.007%	0.017%	0.017%	45.2	47.2	45.5	646	278	268
Amylopectin	0.003%	0.017%	0.016%	45.9	43.5	44.0	1530	256	275
Glycogen	0.006%	0.054%	0.056%	38.8	44.8	46.5	647	83	83
Pullulan	0.16%	2.0%	2.3%	0.102	0.457	0.450	0.0634	0.023	0.020
Maltose ($n=2$)	3.17 mM	3.17 mM	3.15 mM	3.14	3.02	3.22	0.99	0.95	1.02
Maltotriose ($n=3$)	1.30 mM	1.26 mM	1.32 mM	20.0	19.7	20.8	1.54	1.56	1.58
Maltoheptaose ($n=7$)	0.321 mM	0.321 mM	0.30 mM	54.8	57.0	53.2	17.1	17.8	17.7
Amylose EX-1 ($\bar{n}=17$)	0.120 mM	0.145 mM	0.145 mM	48.8	49.9	49.2	40.7	34.4	33.9
Amylose A ($\bar{n}=25$)	0.085 mM	0.124 mM	0.123 mM	46.0	47.2	46.2	54.1	38.1	37.6
Isomaltose ($n=2$)	3.10 mM	3.07 mM	3.27 mM	0.0038	0.0039	0.0034	0.0012	0.0013	0.0011
p-Nitrophenyl α-D-glucoside	4.03 mM	4.18 mM	4.05 mM	0.033	0.034	0.034	0.0081	0.0081	0.0084

n and $\bar{n}$; degree and average degree of polymerization in glucose units, respectively.

denatured at 2 M Gu·HCl or 5 M urea and complete denaturation was achieved with higher concentrations of Gu·HCl (above 4 M) but not with urea. Even with 8 M urea, the decreases in $[\theta]_{218\,nm}$ values of all the enzymes were 60%, more or less, indicating relatively high resistance to urea denaturation.

$Gluc_1$, $Gluc_2$ and $Gluc_3$ were reported to contain 3 residues of half-cystine per mol.[1] Ellman titration[6] of three enzymes detected no free SH groups in the absence of denaturant but detected 1 SH in the presence of 6 M Gu·HCl, indicating that each enzyme contained 1 SH in a buried form, as well as 1 S–S bridge. In the presence of 8 M urea, the buried SH group in each enzyme also became exposed (titratable), although the enzymes were not completely denatured (Fig. 3).

Kinetic Parameters for $Gluc_1$, $Gluc_2$ and $Gluc_3$

The kinetic parameters for the three enzymes were determined at pH 6.0 and 37 °C for various substrates of different sizes: high-molecular-weight soluble starch, amylopectin, glycogen and pullulan, and low-molecular-weight PNPG, isomaltose and malto-oligomers, including maltotriose, maltoheptaose and amyloses EX-1 and A. Table I compares the kinetic parameters for $Gluc_1$, $Gluc_2$ and $Gluc_3$, which were estimated either from Hofstee plots for the high-molecular-weight substrates or from Lineweaver–Burk plots for the low-molecular-weight substrates; each plot appeared to be linear within the limits of probable experimental error.

It was found that $Gluc_2$ and $Gluc_3$ had very similar values of K_m and V_{max} for all the substrates tested. For the low-molecular-weight substrates, the kinetic parameters for $Gluc_2$ and $Gluc_3$ were unchanged from those for $Gluc_1$. As for the malto-oligomers tested, the K_m values of the three enzymes decreased with increasing chain length of substrate in glucose units (n) and the V_{max} values increased with n with a maximum at $n=7$, thus, the V_{max}/K_m values increased sharply with n up to $n=25$. The V_{max} values of the three enzymes for isomaltose with an α-1,6-glucoside linkage were much smaller (about one-thousandth) as compared with those for maltose with an α-1,4-glucoside linkage, while the K_m values differed little for the two substrates.

For the high-molecular-weight substrates, the K_m values of $Gluc_2$ and $Gluc_3$ were consistently higher than those of $Gluc_1$, while the V_{max} values, except for those for pullulan, differed little. The K_m values of $Gluc_2$ and $Gluc_3$ for soluble starch, amylopectin, glycogen and

NII-Electronic Library Service

pullulan were about 2.4, 5.5, 9.2 and 13.4 times higher, respectively, than the corresponding values of $Gluc_1$. Thus, the V_{max}/K_m values of $Gluc_2$ and $Gluc_3$ became considerably lower (about 2.4 to 7.8 times) than the corresponding values of $Gluc_1$ for all the substrates including pullulan, which gave V_{max} values about 4.5 times higher with $Gluc_2$ and $Gluc_3$ than with $Gluc_1$. The K_m and V_{max} values of the enzymes for the high-molecular-weight substrates did not differ greatly, except in the case of pullulan (highly α-1,6-branched), whose K_m and V_{max} values were much higher and lower, respectively, than the corresponding values of the other substrates, so that the V_{max}/K_m value for pullulan is very much the lowest.

Discussion

It is of interest to differentiate and to compare multiple forms of glucoamylases not only from a single source but also from different sources, and in this connection, we have previously isolated two forms of glucoamylase, Gluc M_1 (M.W. 90000) and Gluc M_2 (M.W. 70000), from *Aspergillus saitoi* and carried out comparative characterization.[5,7] Gluc M_1 and Gluc M_2 were found to be in a structural relation similar to the relation among the present *Rhizopus* enzymes, $Gluc_1$, $Gluc_2$ and $Gluc_3$, namely the minor enzyme Gluc M_2 was an enzyme species lacking the C-terminal, but not the N-terminal, glycopeptide moiety of the major enzyme Gluc M_1.

As shown in the present work, $Gluc_2$ and $Gluc_3$ differed little from $Gluc_1$ in the protein backbone structure, although there were some differences in the states of aromatic amino acid side chains (Fig. 1), as was the case with Gluc M_1 and Gluc M_2.[7] Therefore, the N-terminal glycopeptide part of $Gluc_1$, like the C-terminal part of Gluc M_1, seems not to have a critical effect on the gross conformation of the remaining domain of the enzyme. This is not incompatible with the previous finding[3] that $Gluc_1$ has some specific sites within its N-terminal region that are susceptible to the actions of proteinases such as papain and chymotrypsin. Furthermore, $Gluc_2$ and $Gluc_3$ closely resembled $Gluc_1$ in susceptibility not only to heat and extreme pH[1] but also to denaturants (Fig. 3). At present we do not know the role of this flexible or extra N-terminal moiety of $Gluc_1$. On the other hand, Gluc M_2 showed significantly higher susceptibility to denaturants than Gluc M_1, although both enzymes had similar pH and heat stability.[5,7] The different behavior towards denaturants among *Rhizopus* and *Aspergillus* multiple enzyme forms may be ascribed partly, if not completely, to their different contents of S–S bridge; all the *Rhizopus* enzymes had the same number (one) of S–S bridges, whereas Gluc M_2 had 2 less S–S bridges than Gluc M_1 (containing 5 S–S bridges).[7]

The only pronounced difference among $Gluc_1$, $Gluc_2$ and $Gluc_3$ observed in the present work is in the kinetic properties (Table I). $Gluc_2$ and $Gluc_3$ had decreased affinities for all the high-molecular-weight substrates as compared with $Gluc_1$, while retaining the original affinities of $Gluc_1$ for the low-molecular-weight substrates. The degrees of decrease in affinity for $Gluc_2$ and $Gluc_3$ were in the following increasing order for the large substrates: soluble starch < amylopectin < glycogen < pullulan. This order appears to be in parallel with the degree of branching of the substrates. A similar order of decrease in affinity for the large substrates was observed for pap-Gluc and chymo-Gluc[3,8] and for Gluc M_2[7] as compared with the original enzymes, $Gluc_1$ and Gluc M_1, respectively; similar phenomena were also reported by Smiley *et al.*[9] with partially purified preparations of the isoenzymes from *A. niger* and *A. awamori* using fewer substrates. Although Miah and Ueda[10] reported that the three glucoamylases of *A. oryzae* had remarkably different K_m values for soluble starch, their results were dissimilar to those of us and Smiley *et al.*[9] in that the two smaller enzymes had greater affinities for the substrate than the largest enzyme. The reason for this dissimilarity may be resolved when the mutual relationship among the three enzymes of *A. oryzae* is elucidated.

As regards the present K_m values for amyloses EX-1 and A, $Gluc_2$ and $Gluc_3$ had slightly

NII-Electronic Library Service

but significantly larger values than $Gluc_1$. This tendency appears to be more distinct in the case[7] of Gluc M_2 as compared with Gluc M_1. Therefore, amylose EX-1 and/or amylose A may be classifiable as large substrates rather than small substrates, or at least as medium ones. The kinetic results obtained with $Gluc_1$, $Gluc_2$ and $Gluc_3$, together with those with pap-Gluc and chymo-Gluc,[3] strongly suggest that the original enzyme $Gluc_1$ has an additional site(s) interacting with substrates larger than at least amylose EX-1 or amylose A, besides the active center consisting of 6—7 subsites[11] as proposed by Hiromi *et al.*,[12] and that the additional site(s) is probably located within the N-terminal glycopeptide region of about 16000 daltons. Similarly, Gluc M_1 but not Gluc M_2 seems to have such an extra substrate-interacting site(s) in the C-terminal glycopeptide moiety of about 20000 daltons. Nevertheless, it should be noted that the hydrolysis rates of $Gluc_2$ and $Gluc_3$ for the large substrates, except for pullulan, were practically unchanged from those of $Gluc_1$, since the concentration (usually 1%) of each substrate used in the enzyme assay was still much higher than the increased K_m values of $Gluc_2$ and $Gluc_3$.

Further studies are in progress, and preliminary results tend to confirm the presence of an extra binding site in $Gluc_1$ for large substrates, especially for raw starch.

Acknowledgement The authors are greatly indebted to Amano Pharmaceutical Co., for a generous gift of Gluczyme.

References and Notes

1) T. Takahashi, Y. Tsuchida and M. Irie, *J. Biochem.* (Tokyo), **84**, 1183 (1978).
2) T. Takahashi, Y. Tsuchida and M. Irie, *J. Biochem.* (Tokyo), **92**, 1623 (1982).
3) T. Takahashi, Y. Tsuchida, M. Iwama, R. Ohtsuki and M. Irie, *Chem. Pharm. Bull.*, **31**, 1001 (1983).
4) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
5) T. Takahashi, N. Inokuchi and M. Irie, *J. Biochem.* (Tokyo), **89**, 125 (1981).
6) G. L. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).
7) N. Inokuchi, T. Takahashi and M. Irie, *J. Biochem.* (Tokyo), **90**, 1055 (1981).
8) Pap-Gluc (M.W. 57000) and chymo-Gluc (M.W. 64000) were formed from $Gluc_1$ by modification with papain and chymotrypsin, respectively, and were found to lack N-terminal portions of $Gluc_1$ of different sizes.
9) K. L. Smiley, D. E. Hensley, M. J. Smiley and H. J. Gasdorf, *Arch. Biochem. Biophys.*, **144**, 694 (1971).
10) M. N. N. Miah and S. Ueda, *Die Stärke*, **29**, 235 (1977).
11) T. Koyama, N. Inokuchi, Y. Kikuchi, H. Shimada, M. Iwama, T. Takahashi and M. Irie, *Chem. Pharm. Bull.*, **32**, 757 (1984).
12) K. Hiromi, Y. Nitta, C. Numata and S. Ono, *Biochim. Biophys. Acta*, **302**, 362 (1973).

NII-Electronic Library Service