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Proteolytic Modification of a Glucoamylase from a *Rhizopus* sp.

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Three forms of glucoamylase [EC 3.2.1.3] have been purified from a *Rhizopus* sp. and named $Gluc_1$, $Gluc_2$ and $Gluc_3$ in order of content (T. Takahashi *et al.*, *J. Biochem.*, **84**, 1183 (1978)). $Gluc_1$ (M.W. 74000; specific activity 66 units/mg; N-terminal Ala; C-terminal –Ser–Ala·OH) was converted by papain and chymotrypsin into two active derivatives named pap-Gluc and chymo-Gluc, respectively. pap-Gluc was characterized by a molecular weight of 57000 and a specific activity of 88 units/mg, and chymo-Gluc by a molecular weight of 64000 and a specific activity of 78 units/mg. The C-terminal amino acid sequences of both modified enzymes were identical with that of $Gluc_1$ but their N-terminal amino acids were different from that of $Gluc_1$. These results, together with the results of amino acid and sugar analyses, indicate that papain and chymotrypsin liberated glycopeptide and peptide moieties, respectively, from the N-terminal side of $Gluc_1$. The two modified enzymes had almost the same pH optimum, pH stability and heat stability as those of $Gluc_1$. However, they differed from $Gluc_1$ in the values of K_m and V_{max} for high-molecular-weight substrates, although they showed identical kinetic parameters for low-molecular-weight substrates. The close similarity between pap-Gluc and $Gluc_2$ as well as between chymo-Gluc and $Gluc_3$ is discussed.

Keywords——glucoamylase; *Rhizopus*; multiple forms of enzyme; proteolytic modification; papain; chymotrypsin; amino acid composition; kinetic parameter; N-terminal amino acid; C-terminal amino acid sequence

In a previous report we isolated three forms of glucoamylase [EC 3.2.1.3; α-D-(1→4)-glucan glucohydrolase] from a digestive produced from a *Rhizopus* sp. and carried out comparative characterization.[1] The three enzyme forms, designated as $Gluc_1$, $Gluc_2$ and $Gluc_3$ in order of content, differed in both amino acid composition and carbohydrate content and had different molecular weights of 74000, 58600 and 61400, respectively. The three glucoamylases, however, showed common antigenicity in immunodiffusion and close similarity in such enzymatic properties as pH optimum, pH stability, heat stability and divalent cation inhibition. During further studies on the relationship among the three enzymes, $Gluc_1$, the most abundant one, was found to be convertible into two active derivatives similar to $Gluc_2$ and $Gluc_3$ on incubation with certain proteinases. Among the commercially available proteinases tested, papain and chymotrypsin most efficiently converted $Gluc_1$ into $Gluc_2$-like and $Gluc_3$-like enzymes, respectively. This finding may afford a model for studies of the observed multiplicity of the glucoamylase from a *Rhizopus* sp.

The present paper describes the conversion of $Gluc_1$ by papain and chymotrypsin into two active derivatives, and the enzymatic and chemical properties of the proteinase-modified enzymes in comparison with those of $Gluc_2$ and $Gluc_3$ as well as $Gluc_1$.

Materials and Methods

Chemicals——Soluble starch was purchased from Wako Pure Chemicals and used as a substrate after exhaustive dialysis against distilled water. Glycogen (E. Merck), amylopectin (Tokyo Kasei Kogyo Co., Ltd.), maltose (Wako Pure Chemicals) and maltotriose (Nakarai Chemicals, Ltd.) were obtained from the indicated companies. The D-glucose oxidase reagent ("Glucose-Autotest") was obtained from Wako Pure Chemicals, papain (2 times crystallized) was from Sigma Chemical Co., chymotrypsin, trypsin, and carboxy-

peptidases A and B (pretreated with phenylmethylsulfonyl fluoride) from Worthington Biochemical Corp., alkaline protease from Nagase Sangyo Co., Ltd., pronase E from Kaken Kagaku Co., Ltd., and thermolysin (3 times crystallized) from Seikagaku Kogyo Co., Ltd. Ampholine (carrier ampholytes) was purchased from LKB-Produkter. All other chemicals were of analytical reagent grade.

Preparation of $Gluc_1$, $Gluc_2$ and $Gluc_3$——The three forms of glucoamylase were purified from "Gluczyme" (a commercial digestive from a *Rhizopus* sp., Amano Pharmaceutical Co.) according to the method reported previously.[1]

Estimation of Protein——The protein contents of $Gluc_1$, $Gluc_2$ and $Gluc_3$ were estimated using A_{cm}^{280nm} (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 by the D-glucose oxidase method as described previously.[2]

Determination of Kinetic Parameters——The kinetic parameters, K_m and V_{max}, of proteinase-modified glucoamylases were measured as described previously[2] using soluble starch, glycogen and amylopectin as high-molecular-weight substrates, and maltose and maltotriose 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.

Amino Acid Analysis——Samples of about 0.3 mg protein were hydrolyzed with 6 N HCl in evacuated tubes at 110°C. Analyses of 24, 48 and 72 h hydrolysates were performed by the method of Spackman *et al.*[3] with a Nihon Denshi JLC-6AH amino acid analyzer. Estimation of tryptophan was performed by the *p*-dimethylaminobenzaldehyde–H_2SO_4 method.[4] Half-cystine was estimated as cysteic acid after hydrolysis in 6 N HCl of the performic acid-oxidized protein, which was prepared according to the method of Schram *et al.*[5]

Determination of N-Terminal Amino Acid——N-Terminal amino acid was determined by manual Edman degradation as described by Blombäck *et al.*[6] Phenylthiohydantoin (PTH)-amino acid released was identified by high performance liquid chromatography (HPLC) and thin layer chromatography (TLC) as described previously.[7]

Determination of C-Terminal Amino Acid Sequence——C-Terminal amino acid sequence was determined by carboxypeptidase digestion. Each of the proteinase-modified glucoamylases (about 0.1 μmol) which was denatured by heating in distilled water at a concentration of 1 mg/ml at 100°C for 5 min, then incubated with carboxypeptidases A (0.7 units) and B (0.36 units) in 0.05 M Tris-HCl buffer (pH 8.0) at 37°C. Samples of the digestion mixture were taken at intervals to analyze the released amino acids in an amino acid analyzer.

Carbohydrate Analysis——The content of neutral sugar was determined by the phenol–H_2SO_4 method[8] with mannose as a standard. Neutral sugar analysis was performed by the method of Reinhold[9] with a Nihon Denshi JGC-20K gas chromatograph. Amino sugar analysis was performed in an amino acid analyzer after hydrolysis with 4 N HCl in an evacuated tube at 100°C for 8 h.

Polyacrylamide Gel Electrophoresis——Disc electrophoresis in 7.5% polyacrylamide gel, pH 4.3, was performed as described by Reisfeld *et al.*[10] SDS-polyacrylamide gel electrophoresis in 7% gel containing 1% SDS was carried out as described by Shapiro *et al.*;[11] samples were denatured by heating at 100°C for 5 min.

Proteins in the gels were detected by staining with a 0.05% Coomassie brilliant blue G-250 solution in 9% acetic acid containing 45% methyl alcohol.

Isoelectric Focusing——Isoelectric focusing with carrier ampholytes (pH 7—10) was performed in a gradient of glycerol (0—50%, v/v), according to the method of Vesterberg and Svensson.[12] Electrophoresis was conducted in a 110-ml column (LKB-Produkter) at 4°C for 24 h with a final potential drop of 700 V. After termination of electrophoresis, 1.5-ml fractions were collected and the pH values were measured at room temperature. A control run was made without sample and the A_{280nm} of each fraction in the control run was subtracted from that of the sample run.

Results

Digestion of $Gluc_1$ with Various Proteinases

The susceptibility of $Gluc_1$ to various proteinases was preliminarily explored; 1 mg of $Gluc_1$ was incubated at 37°C with 20 μg of each proteinase in 1 ml of the following buffers: 0.1 M Tris-HCl buffer (pH 7.5) for chymotrypsin, trypsin, pronase E, thermolysin and alkaline protease digestions, 0.1 M phosphate buffer (pH 7.2) containing 0.2 mM 2-mercaptoethanol for papain digestion and 0.1 M acetate buffer (pH 5.0) for pepsin digestion. Portions of the digestion mixtures were taken at various times from 0.5—24 h for glucoamylase activity assay and SDS-polyacrylamide gel electrophoresis. The glucoamylase activity of all the proteinase-digestion mixtures changed little during incubation for up to at least 24 h. In contrast, the SDS-polyacrylamide gel electrophoresis patterns of the digestion mixtures changed to different

extents with different proteinases, except in the case of trypsin. The chymotrypsin digestion mixture gave an additional new band of $Gluc_3$-like protein which increased very slowly to about one-fourth of the amount of the unsplit $Gluc_1$ at 24 h. The pepsin digest yielded several new bands with smaller molecular weights in varying proportions with time. For the digests by other proteinases such as papain, pronase E, thermolysin and alkaline protease, no clear bands could be seen throughout the incubation time from 0—24 h. This phenomenon seemed to be ascribable to further action of the proteinases during the SDS denaturation of the digests prior to an electrophoretic run, resulting in the production of small fragments not detectable by this technique. Therefore, the 24-h digests (0.75 ml) obtained with these proteinases were each passed, after concentration, through a column (1×41 cm) of Bio-Gel P-150 to obtain the first-eluted, active peak and the second-eluted, inactive peak; the latter peak contained proteinase as well as the digested fragments. Upon electrophoresis of the individual active peaks thus obtained, the papain digest yielded only one band similar to that of $Gluc_2$ but the pronase E, alkaline proteinase and thermolysin digests gave at least two to four bands including $Gluc_2$-like and (or) $Gluc_3$-like bands.

On the basis of SDS-polyacrylamide gel electrophoresis, $Gluc_1$ appeared to be degraded to smaller proteins with retention of its original activity on incubation with all the proteinases except for trypsin. Among the proteinases used, papain and chymotrypsin seemed to be most suited for the preparation of $Gluc_2$-like and $Gluc_3$-like derivatives, respectively, because the two proteinases did not form any other enzyme forms in the preliminary experiment.

Preparation of $Gluc_2$-like Enzyme

To find the best conditions for the preparation of $Gluc_2$-like enzyme, the time-course of the papain digestion of $Gluc_1$ was followed by SDS-polyacrylamide gel electrophoresis; the digestion was carried out as described above except that a $Gluc_1$/papain ratio of 20: 1 was used. Samples withdrawn at intervals were supplemented with $CuCl_2$[13] at a final concentration of 5 mM to stop papain action during the SDS denaturation of the samples. Within 5 h, most of $Gluc_1$ was converted into $Gluc_2$-like enzyme; at 8 h only one band of $Gluc_2$-like enzyme could be seen and no further change was observed thereafter up to 48 h (Fig. 1a). The glucoamylase activity of the digestion mixture changed little throughout the incubation. Since

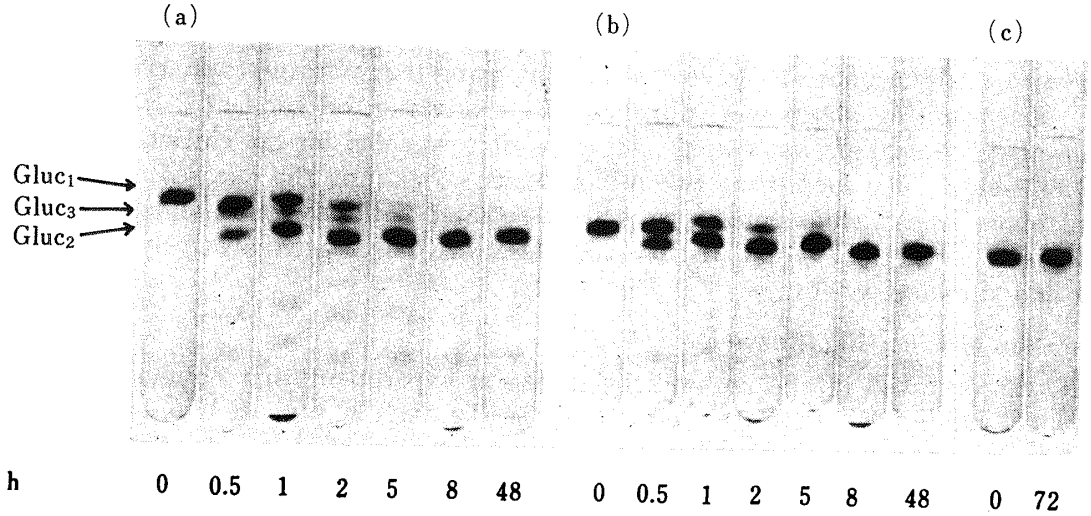

Fig. 1. Time Course of the Papain Digestions of $Gluc_1$(a), $Gluc_3$(b) and $Gluc_2$(c)

One mg of each glucoamylase was incubated with 50 μg of papain in 1 ml of 0.1 M phosphate buffer (pH 7.2) containing 0.2 mM mercaptoethanol at 37°C. One-tenth ml portions of the digestion mixtures were taken at various times, mixed with 0.1 ml of boiling 2% SDS solution containing 50% glycerol and 10 mM $CuCl_2$ and heated in boiling water for 5 min to denature proteins. SDS-polyacrylamide gel electrophoresis was carried out as described in the text; about 15 μg protein was applied to each gel.

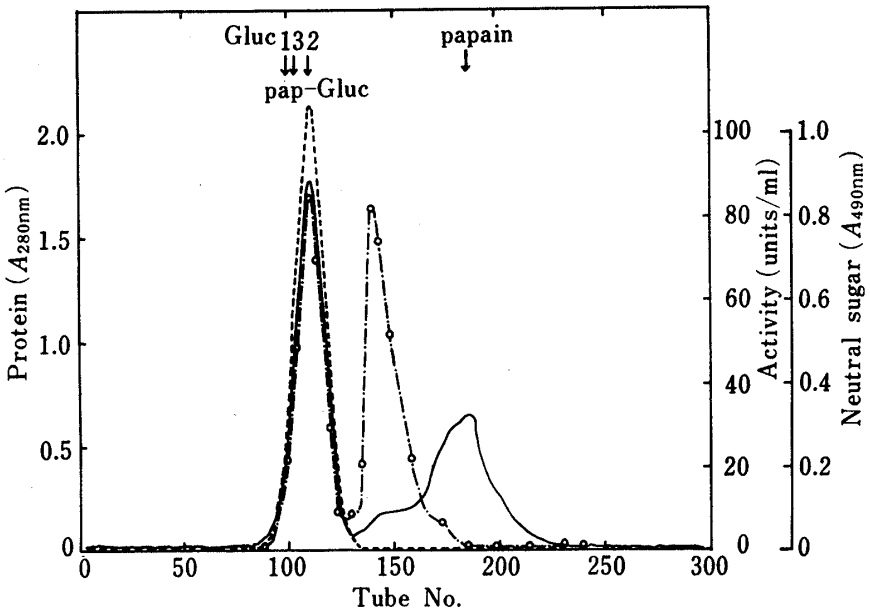

Fig. 2. Gel Filtration on Bio-Gel P-150 of the Papain Digestion Products of $Gluc_1$

The digestion mixture contained 60 mg of $Gluc_1$ and 3 mg of papain in 60 ml of 0.1 M phosphate buffer (pH 7.2) containing 0.2 mM mercaptoethanol. After incubation at 37°C for 24 h, $CuCl_2$ was added to the digestion mixture to a final concentration of 5 mM and the whole was concentrated to about 20 ml. The resulting solution was applied to a column (2.5×95 cm) of Bio-Gel P-150 equilibrated with 0.05 M Tris-HCl buffer (pH 7.0) and the column was eluted with the buffer. Two-ml fractions were collected. Arrows indicate the positions expected for the elution of $Gluc_1$, $Gluc_2$, $Gluc_3$ and also papain. ——, A_{280nm}; ·····, glucoamylase activity; ○—·—○, A_{490nm}.

$Gluc_1$ was converted by papain into $Gluc_2$-like enzyme not directly but *via* a $Gluc_3$-like intermediate as shown in Fig. 1a, the papain digestion of $Gluc_3$ was also undertaken under the same conditions as for the digestion of $Gluc_1$. $Gluc_3$ was found to be digestible with papain to form $Gluc_2$-like enzyme; the digestion reached completion within 8 h (Fig. 1b). Upon incubation of $Gluc_2$ with papain, no further degradation of $Gluc_2$ occurred on the basis of SDS-polyacrylamide gel electrophoresis, even with as long an incubation time as 72 h (Fig. 1c).

For a large-scale preparation of $Gluc_2$-like enzyme, $Gluc_1$ (60 mg) was treated with papain (3 mg) in 0.1 M phosphate buffer (pH 7.2) containing 0.2 mM 2-mercaptoethanol at 37°C for 24 h. The resulting digest was subjected to gel filtration on Bio-Gel P-150 (Fig. 2). The papain-modified enzyme was eluted at the position expected for the elution of $Gluc_2$ but not $Gluc_1$ or $Gluc_3$. The succeeding two minor peaks, containing the released carbohydrate and peptide moieties as well as papain, were not associated with any glucoamylase activity. The active enzyme fraction, designated as pap-Gluc, was dialyzed exhaustively against distilled water and stored at −20°C.

Preparation of $Gluc_3$-like Enzyme

It was observed in the preliminary digestion experiment that chymotrypsin degraded $Gluc_1$ much more slowly than other proteinases such as papain, pronase E and alkaline protease. Therefore, a high ratio of chymotrypsin to $Gluc_1$ of 1: 6 was used; Gluc (60 mg) was treated with chymotrypsin (10 mg) in 60 ml of 0.1 M phosphate buffer (pH 7.2) at 37°C. Under these conditions, most of $Gluc_1$ was converted by chymotrypsin into $Gluc_3$-like enzyme within 40 h, but more prolonged incubation resulted in the appearance of an additional protein which migrated between $Gluc_3$ and $Gluc_2$ in SDS-gel. Thus, the chymotrypsin digestion was terminated at 39 h and the digest was subjected to gel filtration on Bio-Gel P-150 under the same conditions as in Fig. 2. The first-eluted peak possessed glucoamylase activity, the first half

of which contained both $Gluc_3$-like enzyme and a small amount of the undigested $Gluc_1$, while the second half contained only $Gluc_3$-like enzyme. Fractions containing only $Gluc_3$-like enzyme were pooled, dialyzed against distilled water and stored at −20°C. The preparation was designated as chymo-Gluc.

Properties of pap-Gluc and chymo-Gluc

The preparations of pap-Gluc and chymo-Gluc yielded single protein bands in polyacrylamide gel electrophoresis with and without SDS, with mobilities similar to those of $Gluc_2$ and $Gluc_3$, respectively (Fig. 3). SDS-polyacrylamide gel electrophoresis of each modified enzyme yielded a single band of similar mobility in the presence or in the absence of a reducing reagent, demonstrating that the two modified enzymes each consist of a single polypeptide chain as $Gluc_1$ does.[1] Apparent molecular weight was estimated by SDS-polyacrylamide gel electrophoresis calibrated with β-galactosidase, bovine serum albumin, catalase and ovalbumin; the molecular weights of pap-Gluc and chymo-Gluc were found to be 57000 and 64000, respectively, as compared with 73600 for $Gluc_1$.[1]

Each modified enzyme yielded a single symmetrical boundary in ultracentrifugation (not shown here). The sedimentation coefficients ($s_{20,w}$) of the two derivatives were estimated to be 4.31 S.

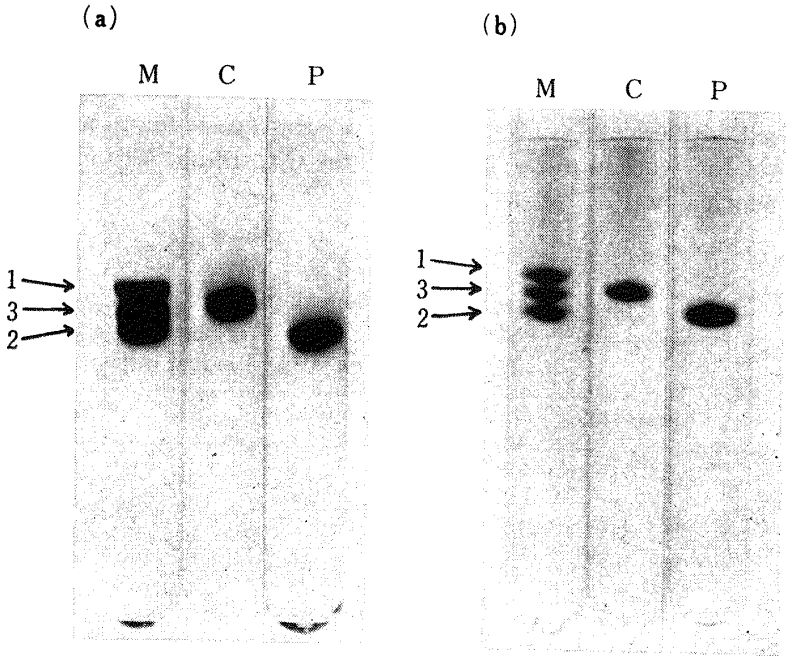

Fig. 3. Polyacrylamide Gel Electrophoresis of pap-Gluc and chymo-Gluc

a) Disc electrophoresis in 7.5% gel at pH 4.3: about 45 μg of enzyme was applied to each gel.
b) SDS-polyacrylamide gel electrophoresis in 7% gel at pH 7.2: about 12 μg of enzyme was applied to each gel. An equi-molecular mixture of $Gluc_1$, $Gluc_2$ and $Gluc_3$ was run under the same conditions for comparison. Symbols: C, chymo-Gluc; P, pap-Gluc; 1, $Gluc_1$; 2, $Gluc_2$; 3, $Gluc_3$.

Isoelectric focusing of the derivatives was carried out in carrier ampholytes, pH 7—10 (Fig. 4). Each derivative yielded a single peak of protein which coincided exactly with that of enzymatic activity. The isoelectric points of pap-Gluc and chymo-Gluc were found to be 8.71 and 8.83, respectively.

The amino acid and sugar compositions of pap-Gluc and chymo-Gluc are summarized in Table I. The analysis results indicate that papain released from $Gluc_1$ a glycopeptide moiety

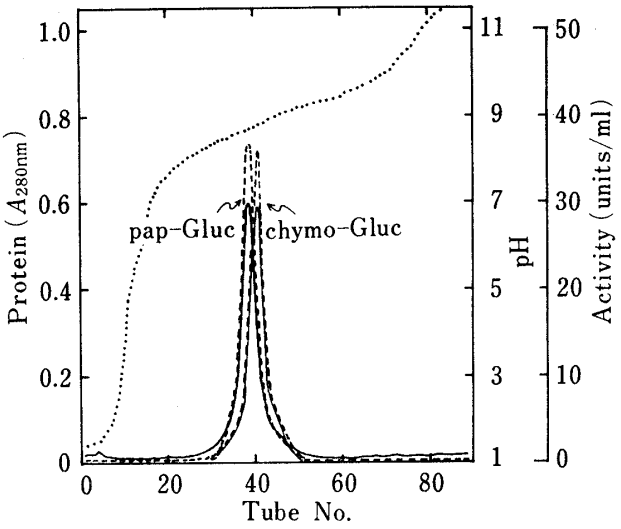

Fig. 4. Isoelectric Focusing of pap-Gluc and chymo-Gluc

The sample applied was about 4—5 mg of enzyme. Isoelectric focusing of each enzyme was conducted separately and the patterns of both modified enzymes are superimposed for comparison. ——, A_{280nm}; ----, glucoamylase activity; ·····, pH.

consisting of about 146 amino acid residues and 19 neutral sugar residues to form pap-Gluc, while chymotrypsin released peptide moiety consisting of about 106 amino acid residues to form chymo-Gluc. Neither papain nor chymotrypsin liberated glucosamine from $Gluc_1$.

The two proteinase-modified enzymes were subjected to manual Edman degradation.[6] pap-Gluc released several PTH-amino acids; chymo-Gluc released mainly PTH–Thr and small amounts of a few other PTH-amino acids. This implies that each preparation is a mixture of several enzyme species having different amino acids as their N-terminal residues. However, no alanine, which is the N-terminal amino acid of $Gluc_1$,[14] was detected as an N-terminal amino acid. On the other hand, pap-Gluc and chymo-Gluc were digested with carboxypeptidases A and B. The digestions permitted us to determine the C-terminal amino acid sequences of the enzymes up to the 2nd amino acid residue; both pap-Gluc and chymo-Gluc were found to have the same C-terminal sequence of –Ser–Ala·OH, the sequence being identical with that of $Gluc_1$.[14]

Table I. Amino Acid and Sugar Compositions of pap-Gluc and chymo-Gluc

Amino acid	Residues per molecule (nearest integer)				
	pap-Gluc	chymo-Gluc	$Gluc_1$[a]	$Gluc_2$[a]	$Gluc_3$[a]
Trp	11	11	13	11	11
Lys	23	25	31	23	26
His	4	4	4	4	4
Arg	14	14	14	14	14
Asp	57	62	78	58	63
Thr	45	51	62	46	53
Ser	54	64	79	55	65
Glu	27	27	34	28	28
Pro	22	25	28	23	27
Gly	43	45	54	44	46
Ala	48	53	65	49	54
Cys 1/2	3	3	3	3	3
Val	29	30	35	30	30
Met	11	11	11	12	11
Ile	21	24	31	21	24
Leu	38	39	37	39	40
Tyr	22	23	37	22	25
Phe	24	25	26	24	25
Mannose	23	41	42	25	43
Glucosamine	8	8	8	8	8

a) Cited from T. Takahashi *et al.*[1,14]

It is of interest to see whether or not such proteolytic modifications of $Gluc_1$ cause any change in enzymatic properties. pap-Gluc and chymo-Gluc hydrolyzed both soluble starch and glycogen almost completely into glucose, as $Gluc_1$ did. The specific activities of pap-Gluc and chymo-Gluc towards soluble starch were determined to be 88 and 78 units/mg, respectively, while that of $Gluc_1$ was 66 units/mg.[1] The starch-hydrolyzing activities of the enzymes were determined as a function of pH; the buffers (0.01 M, I=0.1) used were glycine-HCl buffer for pH 1.0—3.5, acetate buffer for pH 4.0—6.5, borax-HCl buffer for pH 7.0—9.0 and borax-NaOH buffer for pH 9.5—11.0. The modified enzymes showed similar pH-activity profiles with a broad optimum at pH 4.5—5.0.

To test the pH stability, each derivative (0.2 mg/ml) was incubated for 1 h at 37°C in 0.01 M buffer (I=0.1) of various pH values from 2.0—11.0. The modified enzymes were stable between pH 4.0 and 8.0 but lost their activity abruptly at pH values below 3.0 and above 9.0. On heating in 0.01 M acetate buffer (pH 6.0, I=0.1) for 5 min at various temperatures from 25 to 100°C, the enzymes were stable at temperatures up to 50°C but lost their activity completely above 60°C.

The kinetic parameters, K_m and V_{max}, for the modified glucoamylases were determined at pH 6.0 and 37°C; the substrates used were soluble starch, glycogen and amylopectin as high-molecular-weight substrates, and maltose and maltotriose as low-molecular-weight substrates. Table II compares the kinetic parameters for the two modified enzymes with those for the original enzyme. Both pap-Gluc and chymo-Gluc had similar values of K_m and V_{max} for all the substrates tested. The kinetic parameters for the low-molecular-weight substrates of the modified enzyms were little changed from those of $Gluc_1$, but the values of K_m and V_{max}, especially of K_m, for the high-molecular-weight substrates of the modified enzymes were consistently higher than those of $Gluc_1$; the K_m values of the modified enzymes for glycogen, amylopectin and soluble starch were about 9, 5 and 2 times higher, respectively, than the corresponding values of $Gluc_1$.

Table II. Kinetic Parameters for pap-Gluc, chymo-Gluc and $Gluc_1$

Substrate	K_m			V_{max} (units $\times 10^{-2}$/μmol)		
	pap-Gluc	chymo-Gluc	$Gluc_1$	pap-Gluc	chymo-Gluc	$Gluc_1$
Soluble starch	0.012%	0.015%	0.007%	46.4	45.8	37.7
Glycogen	0.054%	0.054%	0.006%	35.7	36.8	30.5
Amylopectin	0.015%	0.017%	0.003%	41.8	41.5	33.5
Maltose	3.35 mM	3.54 mM	3.17 mM	3.1	3.2	2.8
Maltotriose	1.15 mM	1.17 mM	1.31 mM	11.0	10.8	10.4

Discussion

In the present work, the most abundant glucoamylase of a *Rhizopus* sp., $Gluc_1$, was converted by papain and chymotrypsin treatments into two active derivatives named pap-Gluc and chymo-Gluc, respectively. Table III summarizes the properties of pap-Gluc and chymo-Gluc, together with those of $Gluc_1$, $Gluc_2$ and $Gluc_3$.[1] Compared with $Gluc_1$, pap-Gluc and chymo-Gluc were smaller by about 17000 and 10000 daltons in molecular weight, respectively. Nevertheless, the pH-activity, pH-stability and heat stability properties of the modified enzymes were almost exactly the same as those of $Gluc_1$ as well as $Gluc_2$ and $Gluc_3$ (not shown here). It is apparent that the specific activities in terms of units/mg of the two modified enzymes and the three enzyme forms are in inverse relation to their molecular weights, so that they all give similar values for the specific activity in terms of units/mol enzyme. It should be noted, however, that both modified enzymes showed decreased affinities for the high-

TABLE III. Comparison of Properties of pap-Gluc and chymo-Gluc with Those of $Gluc_1$, $Gluc_2$ and $Gluc_3$

	pap-Gluc	chymo-Gluc	$Gluc_1$[a]	$Gluc_2$[a]	$Gluc_3$[a]
Isoelectric point	8.71	8.83	8.70	8.70	8.80
Sedimentation coefficient ($s_{20,w}$)	4.31 S	4.31 S	4.65 S	4.31 S	4.30 S
Molecular weight					
SDS-polyacrylamide gel	57000	64000	73600	59000	66000
Ultracentrifugation	N.D.	N.D.	74000	58600	61400
Chemical composition (residues/mol)					
Amino acid	496	516	642	506	549
Mannose	23	42	42	25	43
Glucosamine	8	8	8	8	8
N-Terminal amino acid	—[b]	—[b]	Ala	Glu	Lys
C-Terminal amino acid sequence	-Ser-Ala·OH	-Ser-Ala·OH	-Ser-Ala·OH	-Ser-Ala·OH	-Ser-Ala·OH
Specific activity (units/mg)	88	78	66	86	79
pH optimum	pH 4—5	pH 4—5	pH 4—5	pH 4—5	pH 4—5
pH stability	pH 4—8	pH 4—8	pH 4—8	pH 4—8	pH 4—8
Heat stability	Up to 50°C	Up to 50°C	Up to 50°C	Up to 50°C	Up to 50°C

a) Cited from T. Takahashi *et al.*[1,14]
b) The N-terminal amino acids of pap-Gluc and chymo-Gluc were not homogeneous. N.D.; not determined.

molecular-weight substrates as compared with $Gluc_1$, while retaining the original affinities of $Gluc_1$ for the low-molecular-weight substrates (Table II). Such decrease in affinity for the large substrates but not for the small substrates was also observed with $Gluc_2$ and $Gluc_3$.[15]

pap-Gluc and chymo-Gluc resemble $Gluc_2$ and $Gluc_3$, respectively, in size, p*I* and enzymatic properties (Table III), kinetic parameters (Table II), and also in amino acid and sugar compositions (Table I). Both modified enzymes had the same C-terminal amino acid sequence as that of $Gluc_1$ but differed in N-terminal amino acid from $Gluc_1$. This indicates that pap-Gluc and chymo-Gluc resulted from the removal of N-terminal glycopeptide and peptide moieties of $Gluc_1$, respectively. Similar results have been obtained with $Gluc_2$ and $Gluc_3$: they have been considered to be derived from $Gluc_1$ by the action of certain proteinases produced by a *Rhizopus* sp. itself.[14,16] In such a conversion of $Gluc_1$ to $Gluc_2$, the liberated N-terminal glycopeptides of molecular weights 16700 and 14400 were isolated in the previous study.[14] In the present work, however, neither glycopeptides nor peptides of comparable size were detectable in the papain and chymotrypsin digests of $Gluc_1$. The only pronounced difference between pap-Gluc and $Gluc_2$ and between chymo-Gluc and $Gluc_3$ was in their N-terminal amino acids; $Gluc_2$ and $Gluc_3$ had glutamic acid and lysine as N-terminal amino acids, respectively,[14] whereas pap-Gluc and chymo-Gluc were heterogeneous with respect to N-terminal residue. The dissimilarity in N-terminal amino acid and the difference in size of the liberated glycopeptides may be attributable to differences in substrate specificity of the proteinases concerned, or merely to differences in the amount of the proteinases present, or to both reasons. It should be noted that pap-Gluc and chymo-Gluc were significantly smaller, although only slightly, than $Gluc_2$ and $Gluc_3$, respectively, as determined by SDS-polyacrylamide gel electrophoresis (Table III).

The present finding that $Gluc_1$ was convertible by several commercial proteinases including papain and chymotrypsin into smaller enzyme species resembling $Gluc_2$ and $Gluc_3$ indicates that $Gluc_1$ has some specific sites susceptible to proteolytic action within the N-terminal region of about 17000 daltons in size, thus confirming the earlier conclusion that the multiplicity of a *Rhizopus* sp. glucoamylase is ascribable to proteolytic modifications of an original enzyme form, $Gluc_1$.[14] The multiplicity of other fungal glucoamylases, *A. awamori* var. *kawachi*[17] and *A. saitoi*[2,7] enzymes, is also ascribed to proteolytic action on an original enzyme: the

former glucoamylase was further shown to be convertible to smaller enzyme forms by the action of some proteinases.[18]

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References and Notes

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