Carbohydrate Research, 203 (1990) 129–138
Elsevier Science Publishers B.V., Amsterdam

Alteration of the properties of *Aspergillus* sp. K-27 glucoamylase on limited proteolysis with subtilisin

Jun-ichi Abe, Kouji Nakajima, and Susumu Hizukuri*
Department of Agricultural Chemistry, Faculty of Agriculture, Kagoshima University, Korimoto-1, Kagoshima 890 (Japan)
Received August 7th, 1989; accepted, in revised form, January 4th, 1990)

ABSTRACT

An active derivative (mol. wt. 48000) of *Aspergillus* sp. K-27 glucoamylase (mol. wt. 76000) was obtained by limited proteolysis with subtilisin. The amino acid sequences of native and modified enzymes at the *N*-termini were Ala-Gly-Gly-Thr-Leu-Asp and Ala-Val-Leu, respectively. The proteolysis greatly decreased the affinity of the enzyme for amylopectin and glycogen, but not for oligosaccharides. It also reduced the ability of the enzyme to degrade raw starch, abolished the ability of the enzyme to adsorb onto starch granules, and eliminated the synergistic action of the enzyme in the hydrolysis of starch granules with α-amylase. These findings imply that the enzyme has a specific affinity site for polysaccharide substrates besides the catalytic site, *i.e.*, a starch-binding site, and that the former is removed by proteolysis. The extent of the reduction in the activity for raw starches caused by the modification varied with the starch source, as the modified enzyme digested raw potato starch better than either raw corn or sweet potato starches. A new method for evaluation of the raw starch-digesting activity of glucoamylase is described.

INTRODUCTION

In a previous paper[1], we reported the properties of glucoamylase and α-amylase produced by *Aspergillus* sp. K-27. The glucoamylase has a strong ability to digest raw starch, and the α-amylase synergistically enhances the digestion with glucoamylase. We suggested the presence on the glucoamylase of a specific affinity site, *i.e.*, a starch-binding site, for large molecular-weight substrates that differs from the active site on the glucoamylase, as well as the involvement of the site in the hydrolysis of raw starch, as was found for *Rhizopus delemar* glucoamylase[2].

We now report the presence and the role of this site of the K-27 glucoamylase in its action, as revealed by limited proteolysis of the enzyme with subtilisin.

EXPERIMENTAL

Materials. — Glucoamylase and α-amylase of *Aspergillus* sp. K-27 were purified from the culture supernatant as previously described[1]. Three forms (GI, GII, and GIII) of glucoamylase of *R. delemar* were purified[2] and G′ was prepared by limited proteolysis

* Author for correspondence.

0008-6215/90/$ 03.50

of GIII with chymotrypsin as previously reported[3]. Subtilisin (Carlsberg and BPN′) was donated by Nagase Sangyo Co. Other proteases were purchased from Sigma Chemical Co. Amylopectins were fractionated under a nitrogen atmosphere with a mixture of 1-butanol and 3-methyl-1-butanol[4].

Wheat, corn, waxy corn, and (hydroxypropyl)ated corn starches were products of Sanwa Starch Co. Ltd. (Nara). Tapioca starch was an imported commercial product. Sweet potato and potato starches were extracted from their respective roots and tubers with ice-cold water by conventional methods, and the prime starch fractions were dried at room temperature.

Enzyme assay. — The enzyme solution (50 μL), suitably diluted with 50mM sodium acetate buffer (pH 4.5) containing 0.02% bovine serum albumin and a 2% soluble starch solution (200 μL) was incubated for 15 min at 45°. The reaction was stopped by heating, and the resultant glucose was determined by the glucose oxidase–peroxidase method[5]. One unit (U) of activity was defined as the amount of enzyme which hydrolyzed 1 μmole of (1→4)-α-D-glucosidic linkage per min under the conditions used.

Analytical methods. — Protein and total carbohydrate were measured by the methods of Lowry *et al.*[6] and Dubois *et al.*[7], respectively. The extent of β-amylolysis of polysaccharides was determined by the method of Takeda and Hizukuri[8]. The average chain length ($\overline{CL}$) of a substrate was calculated from the amount of non-reducing, terminal residue, as determined by modified Smith degradation, followed by the glycerol assay[9] and assay for total carbohydrate. The average exterior-chain length ($\overline{ECL}$) of the substrate was calculated as follows:

$$\overline{ECL} = \overline{CL} \times \beta\text{-amylolysis limit} + 2.0$$

N-Terminal, amino acid sequencing was performed by an Applied Biosystems model 470A gas-phase sequencer, and the PTH-amino acids were identified on an Applied Biosystems PTH Analyzer, model 120A.

Proteolysis of glucoamylase with subtilisin. — *Aspergillus* sp. K-27 glucoamylase (*G*, 35 mg) was digested with subtilisin (7 mg) in 50mM Tris–HCl buffer (5 mL), containing 2mM calcium chloride, pH 7.5 for 48 h at 37°.

RESULTS AND DISCUSSION

Preparation of subtilisin-modified glucoamylase. — The *Aspergillus* sp. K-27 glucoamylase (G) was treated with one-fifth its weight of various proteases, and its activity and molecular weight were monitored in order to determine whether partial proteolysis occurred without any loss of the activity for soluble starch. No proteolysis, or only a little proteolysis, was shown to occur with *A. saitoi* protease, chymotrypsin, papain, subtilisin (BPN′), thermolysin, and trypsin by SDS-gel electrophoresis, but subtilisin (Carlsberg) converted G to a new form that migrated faster than the native enzyme as determined by SDS-gel electrophoresis. The new form, corresponding to 48 000 daltons, appeared on 3-h incubation with the subtilisin. Almost all G was converted to the new form upon 24-h incubation, and no further change occurred

during incubation for a further 24 h. The other fragment(s) released by this proteolysis appear to be degraded into small molecules, as no such fragments were detected by electrophoresis. The activity for soluble starch under the standard assay conditions remained constant during the proteolysis. The modified glucoamylase (mG) was recovered from the reaction mixture by affinity chromatography on cyclomaltoheptaose-coupled Sepharose 6B[1] (column size 1 × 39 cm) as follows. After the addition of the reaction mixture onto the column, the column was washed with 10mM acetate buffer (pH 4.5) until no proteins eluted. The column was then eluted stepwise with increased concentrations of cyclomaltoheptaose containing the buffer (0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 $mg.mL^{-1}$). The protease and α-amylase were in the effluent, and mG was eluted with the highest concentration of cyclomaltohexaose. The mG-containing fractions were collected, concentrated, and further purified on a column of Bio-Gel P-100. A minute amount of α-amylase, which was found in the subtilisin preparation by a sensitive assay of using (hydroxypropyl)ated starch[10], was not detectable in mG. The modified glucoamylase thus obtained was found to be homogenous on both SDS-gel (Fig. 1) and disc-gel electrophoresis.

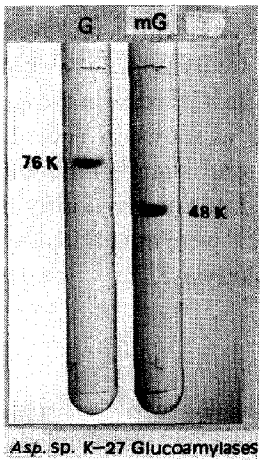

Fig. 1. SDS-polyacrylamide gel-electrophoresis of G and mG. Each protein (5 mg) was electrophoresed by the method of Weber and Osborn (ref. 27), and then stained with Coomassie brilliant blue R-250.

Properties and action of the glucoamylases on water-soluble substrates. — The mol.wt. of mG was estimated to be 48 000 by SDS-gel electrophoresis, a value which was 63% of that of the original enzyme. The specific activity of the modified glucoamylase (6.48 $U.nmole^{-1}$) was the same as that of the native enzyme, indicating that a part not involved in the catalytic action is processed, and that a part containing the active site is inert to proteolysis. The released part, however, contributed slightly to the thermal stability, the t_m being decreased by 3–4° due to the modification (Fig. 2).

The mG showed slightly higher K_m and V_{max} values for oligosaccharides than G (Table I), as well as similar subsite affinities[11], which were calculated from the values for linear maltooligosaccharides (Fig. 3). The kinetic parameters of both G and mG for

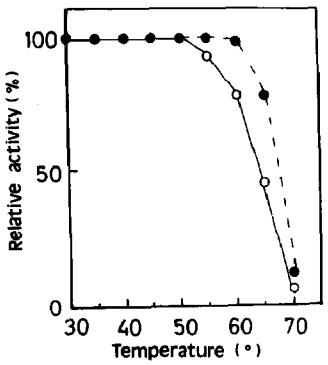

Fig. 2. Thermal stability of G and mG. Each enzyme solution in 50mM acetate buffer (pH 4.5) was incubated for 15 min at the various temperatures indicated, and then the remaining activity was determined. ●, G; ○, mG. The activity of the non-incubated enzyme was taken as 100.

panose were almost the same, indicating that these enzymes have similar activities for not only (1→4)-*α*- but also for (1→6)-*α*-D-glucosidic linkages. The above results further suggest that the active site remained intact during the proteolysis, which removed one-third of G. However, the rates of hydrolysis of glycogen and pullulan at 0.25% were decreased by the modification (Fig. 4). This decrease is due to the great increase in the K_m values (38–1730 fold) for these high molecular-weight substrates, as shown in Table I, while the V_{max} values of mG for polysaccharides were similar or only slightly different (0.9 1.7 fold). The limits of hydrolysis[2,10] of glycogen (97%) and potato amylopectin (80%) due to the presence of small amounts of phosphate esters[12,13] were not altered by the proteolysis, suggesting G and mG were entirely free from *α*-amylase.

TABLE I

Kinetic constants of *Aspergillus* sp. K-27 glucoamylases for various oligosaccharides and polysaccharides

Substrate	*G*		*mG*	
	K_m^a	*Molecular activity*[b]	K_m	*Molecular activity*
Oligosaccharides				
Maltose	1.3	900	1.3	830
Maltotriose	0.45	3900	0.44	2500
Maltotetraose	0.18	4400	0.24	6200
Maltopentaose	0.17	5000	0.21	7400
Maltohexaose	0.15	6200	0.19	7300
Maltoheptaose	0.12	6100	0.17	7900
Panose	56	1200	72	1200
Polysaccharide				
Potato amylopectin	0.0002	5200	0.13	8200
Rabbit liver glycogen	0.0003	6700	0.52	5800
Waxy rice amylopectin *β*-LD	0.02	3300	0.75	3700

[a] mM (non-reducing, terminal residue). [b] $mole.min^{-1}.mole^{-1}$.

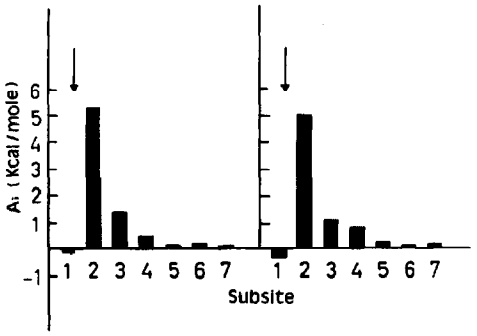

Fig. 3. Histograms of the subsite affinities of G (left) and mG (right). The subsite affinities were calculated from the results in Table I by the method of Hiromi *et al.* (ref. 11). The arrow shows the position of the catalytic site.

Thus, the partial proteolysis decreased the affinity of the enzyme for polysaccharides without having any adverse effect on the affinity for oligosaccharides, as was observed for *R. delemar* glucoamylase[2], indicating that the part released by the subtilisin is in the domain containing the starch-binding site or the site itself, which participates in the binding to the polysaccharides.

The kinetic parameters (Table II) of mG for amylopectins of various $\overline{\text{ECL}}$ suggest that the K_m, but not the V_{max}, value correlates well with $\overline{\text{ECL}}$. Plots (Fig. 5) of the degree of polymerization (d.p.) or $\overline{\text{ECL}}$ against the K_m values (Tables I and II) gave two parallel, linear relationships; K_m (mM) = $(-0.0230) \times$ d.p. $+ 0.329$ (I), r = 0.994 in the range of d.p. 4–7, and K_m (mM) = $(-0.0230) \times \overline{\text{ECL}} + 0.470$ (II), r = 0.996 in range of $\overline{\text{ECL}}$ 12.9–16.7. The K_m values for waxy rice amylopectin and maltoheptaose were equal, although the $\overline{\text{ECL}}$ of the former is greater than that of the latter. This suggests that amylopectin of waxy rice is recognized by the enzyme as a linear substrate of d.p. 7, and the shift of the line for equation (2) from that for equation (1) (Fig. 5) is probably due to the steric hindrance caused by the branch linkage. The other amylopectins of $\overline{\text{ECL}}$ 13.4–16.7 correspond to linear substrates of d.p. 7–11, as judged from the K_m

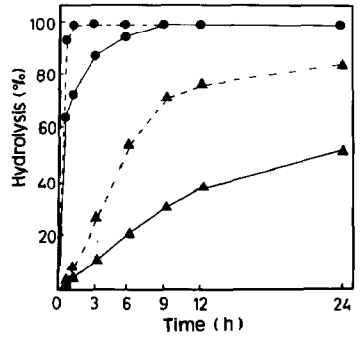

Fig. 4. Hydrolysis of glycogen and pullulan by G (----) and mG (———). The reaction mixture contained 0.25% substrate, 50mM sodium acetate buffer (pH 4.5) and 5 U of glucoamylase, in a final volume of 2 mL, and was incubated at 45°. ●, glycogen; ▲, pullulan.

TABLE II

Kinetic constants of mG for amylopectins with various chain lengths

Amylopectin	$\overline{ECL}$	$\overline{CL}$	K_m[a]	*Molecular activity*[b]
Yamanoimo(Yam)	16.7	25.4	0.083	6200
Potato	15.0	24.3	0.13	8200
Corn	13.4	20.0	0.16	7300
Waxy rice	12.9	18.6	0.17	6600

[a] mM (non-reducing, terminal residue). [b] mole.min^{-1}mole^{-1}.

values in Table II and equation (1), suggesting that the hindrance extends over six glucosyl residues.

Action of glucoamylases on raw starch. — The enzymic activity for raw starch was decreased, and the relative activity toward various raw starches was altered (Table III) by the proteolytic modification. Partial proteolysis decreased the activities for potato, sweet potato, tapioca, corn, and waxy corn starches to 67%, 42%, 43%, 33%, and 39%, respectively, of the original activities. Consequently, mG showed higher activity for potato starch than for corn starch. Such an enzyme has not been previously reported. This implies that granules of corn and potato starch, which are organized as two different structures, are each digested in a different manner. It is of great interest that the proteolysis changes the specificity of the enzyme for raw starches, and potato starch is the best substrate for the resultant enzyme, mG.

As reported previously[1], G shows a synergistic action on raw starch with α-amylases of various origins; however, mG did not act synergistically, because, for example, mG and K-27 α-amylase hydrolyzed 13% and 6%, respectively, raw corn starch, and 19% was hydrolyzed through the concomitant action of the two enzymes. Thus, the previous finding[1] that the starch-binding site was required for the synergistic degradation of raw starch granules with α-amylase was also observed in this case.

More than 98% of G was adsorbed on raw wheat starch, while, on the other hand, only 1% of mG was adsorbed under the same conditions, *i.e.*, when 7 U of the enzymes

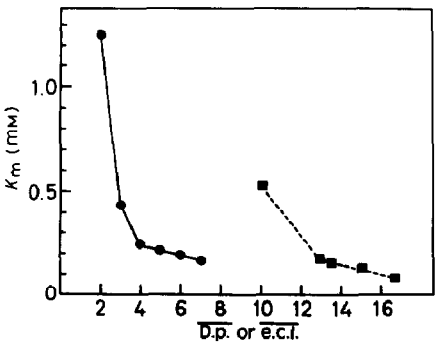

Fig. 5. Relationship between DP of $\overline{ECL}$ and K_m values.

TABLE III

Relative activities[a] of glucoamylases for raw starches

Starch	*G*	*mG*
Potato	60	40
Sweet potato	72	30
Tapioca	112	48
Corn	100	30
Waxy corn	216	85

[a] Initial velocity under the conditions described below and that of G for corn starch was taken as 100. Each reaction mixture (5 mL) containing 250 mg of raw strarch and 20 U of G or mG in 50mM sodium acetate buffer (pH 5.5)–2mM $CaCl_2$, was incubated at 45° for 1 h with mild stirring.

were incubated for 20 min at 4° with 200 mg of raw wheat starch suspension in 50mM sodium acetate buffer (pH 4.5). No degradation of starch occurred due to low temperature and the short incubation period. Obviously, the loss of affinity for polysaccharides in solution accompanied the decrease in the adsorption on and digestion of raw starches, suggesting that the starch-binding site is also involved in digestion of water-insoluble substrates, *i.e.*, raw starches, as observed in the cases or *R. delemar* glucoamylase[2,3] and *Rhizopus* sp.[14]. The starch granules tended to make a slimy aggregate, and the particles settled rapidly from aqueous suspension in the presence of G, but not in the presence of mG (Fig. 6). This phenomenon, which was a novel observation, seems to result from the association of the granules through the starch-binding site.

Hayashida and his co-workers reported[15–18] that glucoamylase from *Aspergillus awamori* var. *kawachi* lost both the abilities of raw-starch digestion and of adsorption on granules upon proteolytic digestion. We found, upon proteolysis, the almost complete loss of adsorption onto raw starch, but only a partial loss of the ability of digestion. Therefore, we consider that the high ability of the enzyme for adsorption onto starch is responsible for the strong ability for the digestion of raw starch; however, the adsorption of the enzyme on starch granules may not be required for the digestion. In the case of α-amylase, Hayashida *et al.*[19] have found a non-adsorbable enzyme which degrades raw starch. Svensson *et al.*[20,21] pointed out that the region near the *C*-terminus of *A. niger* glucoamylase G1, which is different from the active site, was concerned in the adsorption of the enzyme onto starch granules, and that two tryptophan residues in the region were essential for this action. They also mentioned that the region was not found in glucoamylase G2 (a mixture of two forms), which was thought to be derived by the limited proteolysis of the *C*-terminus (102 or 104 amino acid residue) of glucoamylase G1. Recently Svensson *et al.*[22] described that the sequence of this region was well conserved among various amylases which were adsorbable onto starch granules. Hayashida *et al.*[23,24] reported on *A. awamori* var. *kawachi* G1 that the carbohydrate region of the raw starch affinity site plays an important role in the digestion and adsorption onto raw starch. According to amino acid sequences of *A. niger* glucoamylases G1 and G2 (refs. 20 and 21), however, the region described is identical to the

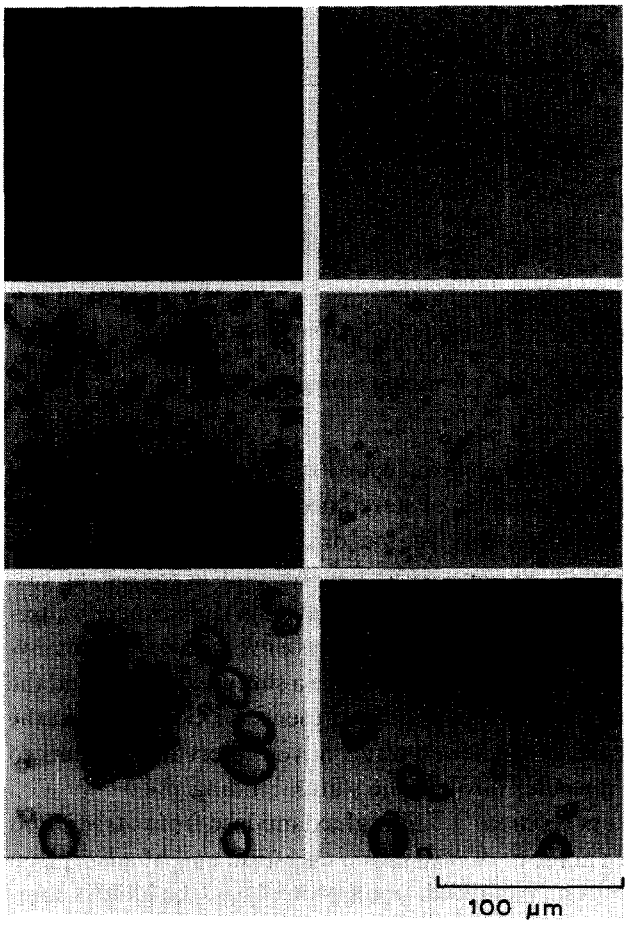

Fig. 6. Light micrographs of various starches after incubation with G (left) and mG (right). A mixture of 10 U of enzyme and 200 mg of starch granules was incubated at 4°. A, wheat starch; B, corn starch; C, potato starch.

C-terminus of glucoamylase G2, which is known not to be able to digest and adsorb onto raw starch. Now we are studying the interaction between the starch-binding site and starch granules.

The *N*-terminal amino acid sequences of G and mG were found to be Ala-Gly-Gly-Thr-Leu-Asp and Ala-Val-Leu, respectively. This implies that G has a different *N*-terminal structure from that (Ala-Thr-Leu-Asp-Ser-Try-Lew) of *A. niger*[25] and *A. awamori*[26] and that the *N*-terminal peptide portion was processed with subtilisin; however, it is not clear as yet whether the peptide portion involves the starch-binding site. Studies on these aspects are presently being carried out.

Rapid evaluation of glucoamylase for raw-starch digestibility. — The enzyme with a low K_m for polysaccharides has strong raw-starch digesting activity. Therefore, the relative activities at suitably high and low concentrations of polysaccharide substrate may allow reasonable estimation for the raw-starch digestibility of the enzyme. In fact,

TABLE IV

Relative activities[a] of glucoamylases with low and high concentrations of polysaccharide substrates

Substrate	*G*	*mG*
Amylopectin		
Yamanoimo	96.8	16.4
Potato	90.0	12.2
Corn	96.3	16.5
Waxy rice	93.2	12.2
Glycogen		
oyster	97.7	5.6

[a] Ratios of the activities with 0.01% and 1% of the substrates.

G showed almost the same activities at a low concentration (0.01%) as at a high concentration (1%) of amylopectins and glycogen, while mG showed only 12.2 and 5–6% activities, respectively, at the low substrate concentration, as shown in Table IV. Thus, comparison of the relative activities for glycogen (oyster) at 1% and 0.01% allow a simple estimation for the raw-starch digesting activity of a glucoamylase, and such may be whorthwhile to attempt for other enzymes. Table V shows another example for the application of this method to *R. delemar* enzymes. Component GIII (ref. 2), which has the starch-binding site and strong raw-starch digesting activity, showed 89% relative activity at the low substrate concentration, while the others, which lack the site and have only weak starch-digesting activity, showed only 5–6% of the original activity. Thus, this seems to be an easy method for the evaluation of the raw-starch digesting-activity of a glucoamylase.

ACKNOWLEDGMENTS

This work was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan to J. A. (Grant No. 63760092), and Daikin Industries, Ltd. (Osaka).

TABLE V

Relative activities of various forms of *Rhizopus delemar* glucoamylase at low and high glycogen concentrations

Enzymes	*Relative activity*[a]
GI	5.4
GII	5.3
GIII	89.2
G′	5.6

[a] Ratio of the activities for 0.01% and 1% oyster glycogen.

REFERENCES

1 J. Abe, K. Nakajima, H. Nagano, and S. Hizukuri, *Carbohydr. Res.*, 175 (1988) 85–92.
2 J. Abe, H. Nagano, and S. Hizukuri, *J. Appl. Biochem.*, 7 (1985) 235–247.
3 J. Abe and S. Hizukuri, *Denpun Kagaku*, 35 (1988) 43–47.
4 S. Hizukuri, Y. Takeda, M. Yasuda, and A. Suzuki, *Carbohydr. Res.*, 94 (1981) 205–213.
5 D. M. Kilburne and P. M. Taylor, *Anal. Biochem.*, 27 (1969) 555–558.
6 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
7 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
8 Y. Takeda and S. Hizukuri, *Biochim. Biophys. Acta*, 185 (1969) 469–471.
9 S. Hizukuri and S. Osaki, *Carbohydr. Res.*, 63 (1978) 261–264.
10 J. Abe, Y. Takeda, and S. Hizukuri, *Biochim. Biophys. Acta*, 703 (1982) 26–33.
11 K. Hiromi, Y. Nitta, C. Numata, and S. Ono, *Biochim. Biophys. Acta*, 302 (1973) 362–375.
12 S. Tabata, K. Nagata, and S. Hizukuri, *Starch/Stärke*, 27 (1975) 333–335.
13 J. D. Fontana, *FEBS Lett.*, 109 (1980) 85–92.
14 T. Takahashi, K. Kato, and Y. Ikegami, *J. Biochem.*, 98 (1985) 663–671.
15 S. Hayashida, T. Nomura, E. Yoshino, and M. Hongo, *Agric. Biol. Chem.*, 40 (1976) 141–146.
16 E. Yoshino and S. Hayashida, *J. Ferment. Technol.*, 56 (1978) 289–295.
17 S. Hayashida and E. Yoshino, *Agric. Biol. Chem.*, 42 (1978) 927–933.
18 P. Q. Flor and S. Hayashida, *Appl. Environ. Microbiol.*, 45 (1983) 905–912.
19 S. Hayashida, Y. Teramoto, and T. Inoue, *Appl. Environ. Microbiol.*, 54 (1988) 1516–1522.
20 B. Svensson, A. J. Clarke, and I. Svendsen, *Carlsberg Res. Commun.*, 51 (1986) 61–73.
21 B. Svensson, K. Larsen, and A. Gunnarsson, *Eur. J. Biochem.*, 154 (1986) 497–502.
22 B. Svensson, H. Jespersen, M. R. Sierks, and E. A. MacGregor, *Biochem. J.*, 264 (1989) 309–311.
23 S. Hayashida, K. Nakahara, K. Kuroda, T. Miyata, and S. Iwanaga, *Agric. Biol Chem.*, 53 (1989) 135–141.
24 S. Hayashida, K. Nakahara, W. Kanlayakrit, T. Hara, and Y. Teramoto, *Agric. Biol. Chem.*, 53 (1989) 143–149.
25 B. Svensson, K. Larsen, I. Svendsen, and E. Boel, *Carlsberg Res. Commun.*, 48 (1983) 529–544.
26 J. H. Nunberg, J. H. Meade, G. Cole, F. C. Lawyer, P. McCabe, V. Schweickart, R. Tal, V. P. Wittman, J. E. Flatgaard, and M. A. Inis, *Mol. Cell. Biol.*, 4 (1984) 2306–2315.
27 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406–4412.