APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1994, p. 3926–3930
0099-2240/94/$04.00+0

Vol. 60, No. 11

Analysis of the Raw Starch-Binding Domain by Mutation of a Glucoamylase from *Aspergillus awamori* var. *kawachi* Expressed in *Saccharomyces cerevisiae*

MASATOSHI GOTO,* TSUYOSHI SEMIMARU, KENSUKE FURUKAWA, AND SHINSAKU HAYASHIDA

Laboratory of Applied Microbiology, Department of Agricultural Chemistry, Kyushu University, Fukuoka 812, Japan

Received 21 March 1994/Accepted 9 August 1994

Carboxy-terminal deletions were introduced into the raw starch-binding domain (A-515 to R-615) encoded by the gene for glucoamylase I (GAI) from *Aspergillus awamori* var. *kawachi*. Genes coding for proteins designated GA596 (A-1 to E-596), GA570 (A-1 to A-570), and GA559 (A-1 to N-559) were constructed and resulted in truncated proteins. All of the mutant genes were expressed heterologously in *Saccharomyces cerevisiae*. GA596 adsorbed to raw starch and digested it. GA570 and GA559 did not adsorb to raw starch or to an α-cyclodextrin–Sepharose CL-4B gel under our experimental conditions. However, GA570 was able to digest raw starch, and the digestion of raw starch by GA570 was inhibited by β-cyclodextrin. Residue Trp-562 of GAI, which was suggested previously to contribute to formation of an inclusion complex with raw starch, was replaced by Leu (GAW562L), Phe (GAW562F), and Gly (GAW562G). GAW562L and GAW562F adsorbed to raw starch and an α-cyclodextrin gel, but GAW562G did not. Although GAW562L digested raw starch to the same extent as wild-type GAI (designated GAY), GAW562F and GAW562G exhibited less ability to digest raw starch. On the basis of our results, it appears that the sequence around Trp-562, PL(W-562)YVTVTLPA, is the minimal sequence necessary for digestion of raw starch and that hydrophobic residue Trp-562 contributes to formation of an inclusion complex. The sequence near Trp-589, which has abundant hydrogen bond-forming residues and the charged amino acid residues needed for stable adsorption to raw starch, probably assists in the formation of the inclusion complex.

Aspergillus awamori var. *kawachi* glucoamylase I (GAI) digests both soluble starch and insoluble raw starch. GAI is composed of two domains (2, 6), the catalytic GAI′ region near the amino terminus (A-1 to V-469) and the raw starch affinity site near the carboxy terminus (A-470 to R-615), which is essential for digestion of raw starch because of its specific ability to adsorb to it. Furthermore, the raw starch affinity site can be divided into the Gp-I region (A-470 to V-514) and the Cp region (A-515 to R-615) (the raw starch-binding domain) on the basis of differences in the functions of the two regions (1, 2, 9).

The Gp-I region is composed mainly of Thr and Ser residues linked to a short mannoside moiety (5, 9), which promotes the digestion of raw starch (3, 8).

The Cp region has the ability to bind to raw starch, as well as to α-cyclodextrin (α-CD), β-CD, and γ-CD, the structures of which resemble the helical structure of the amylose that makes up raw starch (11); α-CD, β-CD, and γ-CD are all specific inhibitors of binding to and digestion of raw starch by GAI (2). We previously reported that the Cp region of GAI stereospecifically recognizes the structure of the secondary OH side of β-CD and that binding of the Cp region to cyclodextrins and raw starch is accomplished via formation of an inclusion complex between residue Trp-562 and a hydrophobic cavity in the substrate (4).

In this paper we describe the properties of carboxy-terminally deleted GAIs and GAIs with substitutions for Trp-562 and Trp-589, which were generated as part of an attempt to identify the minimal necessary sequence of the Cp region and the residues that form the inclusion complex that is essential for binding to raw starch and digestion of raw starch by GAI.

* Corresponding author. Phone: 81-092-641-1101, ext. 6193. Fax: 81-092-632-1960.

MATERIALS AND METHODS

Microorganisms and plasmids. Plasmids YEUp3 and pCSα01 (13) were kindly provided by F. Hishinuma. *Escherichia coli* JM109 was used as the host strain for general genetic manipulations, and *E. coli* BMH71-18 (*mutS*) was used as the host strain for site-directed mutagenesis. *Saccharomyces cerevisiae* CG379 (*MATα ura3 leu2 trp1 his3*) was used as the host strain for expression of mutant genes for GAI. *S. cerevisiae* was grown at 30°C in medium that contained (per liter) 20 g of glucose, 6.7 g of yeast nitrogen base without amino acids (Difco, Detroit, Mich.), 20 mg of tryptophan, 20 mg of histidine, and 30 mg of leucine. YPD medium, which contained (per liter) 20 g of glucose, 10 g of yeast extract, and 20 g of Polypeptone, was used for large-scale production of enzymes in recombinant yeast strains.

Construction of cDNAs for carboxy-terminally deleted variants of GAI. cDNAs for full-length and carboxy-terminally deleted GAI of *A. awamori* var. *kawachi* that were suitable for expression in yeast cells were amplified by the PCR. An oligonucleotide whose sequence corresponded to the 5′ sequence of GAI cDNA (7), 5′-ACAGAATTCAAGCTTCTGC GACCTTGGATTCGTGG-3′, was used as the forward primer (the *Eco*RI site is underlined with one line, the *Hin*dIII site is underlined with two lines, and the extra CT nucleotides are indicated by boldface type). Four oligonucleotides, 5′-AAGA ATTCAAGCTTCTACCGCCAGGTGTCGGT-3′ for GAY (wild-type GAI, A-1 to R-615), 5′-AAGAATTCAAGCTTC TATTCCCGGTTCGGGTCGCT-3′ for GA596 (A-1 to

E-596), 5′-AAGAATTCAAGCTTCTAAGCCGGCAGAGT CACAGT-3′ for GA570 (A-1 to A-570), and 5′-AAGAATTC AAGCTTCTAGTTACTGGAAGTGTACTT-3′ for GA559 (A-1 to N-559), were used as reverse primers (in each oligonucleotide the *Eco*RI site is underlined with one line, the *Hin*dIII site is underlined with two lines, and the termination stop codon is indicated by boldface type). Each reaction was performed in a total volume of 50 μl that contained PCR buffer (Promega), deoxynucleoside triphosphates (each at a concentration of 100 μM), 1 μM forward primer, 1 μM reverse primer, 0.5 U of *Taq* DNA polymerase (Promega), and 50 ng of plasmid pUC118-GAI (7) as the template DNA. The desired DNAs were amplified for 20 cycles under the following conditions: denaturation at 95°C for 30 s; primer annealing at 55°C for 30 s; and primer extension at 72°C for 1.5 min. The amplified DNAs were digested with *Eco*RI and were introduced into the *Eco*RI site of pUC19 to generate pUC-GAY, pUC-GA596, pUC-GA570, and pUC-GA559.

Site-directed mutagenesis of GAI. To convert W-562 of GAI to L-562, F-562, and G-562 and to convert W-589 to Q-589, four oligonucleotides (5′-AGTTACATACAAAAGCGGGT T-3′ for L-562, 5′-CAGTTACACTAGAAAAGCGGGTTA-3′ for F-562, 5′-CAGTTACATAGCCAAGCGGGTTACT-3′ for G-562, and 5′-TTCGGGTCGCTCTCTTGCTCCACGGAG TCA-3′ for Q-589) were used as mutagenic primers (the nucleotides corresponding to W-562 and W-589 are underlined with one and two lines, respectively, and the newly inserted nucleotides are indicated by boldface type), and pUC-GAY was used as the template DNA to generate pUC-GAW562L, pUC-GAW562F, pUC-GAW562G, and pUC-GAW589Q. Site-directed mutagenesis was carried out by using a U.S.E. mutagenesis kit (Pharmacia, Uppsala, Sweden) according to the protocol provided by the supplier. The mutations were confirmed by DNA sequencing.

Construction of yeast vectors for expression and secretion of glucoamylases. YEUp3 was digested with *Hin*dIII, filled in, and self-ligated to construct YEUp3H, which lacked a *Hin*dIII site. The *Eco*RI fragment containing the promoter sequence, signal sequence, and terminator sequence of the *MFα1* gene from pSCα01 (13) was introduced into the *Eco*RI site of YEUp3H to generate the secretion plasmid, YEUp3Hα. Yeast secretion vectors (namely, YEUp-GAY, YEUp-GA596, YEUp-GA570, YEUp-GA559, YEUp-GAW562L, YEUp-GAW-562F, YEUp-GAW562G, and YEUp-GAW589Q) (Fig. 1) were constructed by inserting a *Hin*dIII fragment that included a mutant gene for glucoamylase from a pUC-GA plasmid into YEUp3Hα. Yeast cells were transformed by electroporation performed with a model BTX 600 system (Biotechnologies & Experimental Research Inc., San Diego, Calif.) according to the protocol provided by the supplier.

Purification of enzymes. Filtrates from recombinant yeast cell cultures were brought to 60% (wt/vol) saturation with ammonium sulfate. Then GAY (wild-type GAI), GA596, GAW562L, GAW562F, and GAW589Q were purified by column chromatography on a Sephacryl S-200 column (Pharmacia) equilibrated with 0.05 M acetate buffer (pH 4.5) containing 0.1 M NaCl, α-CD–Sepharose CL-4B (4), and TSK-G3000SW (Tohso, Tokyo, Japan) equilibrated with 0.1 M phosphate buffer (pH 6.0) containing 0.1 M Na_2SO_4. GA570, GA559 and GAW562G were purified by column chromatography on a Sephacryl S-200 column equilibrated with 0.05 M acetate buffer (pH 4.5) containing 0.1 M NaCl and butyl-Toyopearl 650S (Tohso) equilibrated with 30% $(NH_4)_2SO_4$; the glucoamylases were eluted with 10% $(NH_4)_2SO_4$ and TSK-G3000SW equilibrated with 0.1 M phosphate buffer (pH 6.0) containing 0.1 M Na_2SO_4.

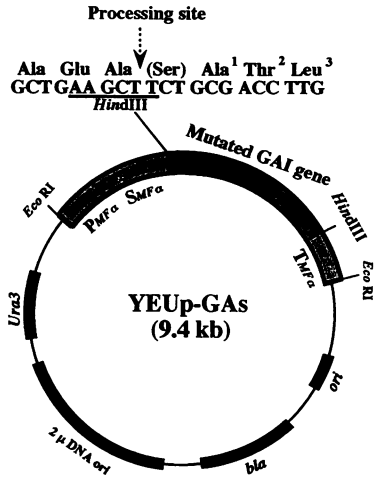

FIG. 1. Yeast secretion vectors that include the wild-type gene for GAI and various mutant genes for glucoamylase. Wild-type and mutant glucoamylases were expressed in yeast cells as precursor proteins, and mature glucoamylases that had an extra Ser residue at the amino terminus were secreted after the preproglucoamylases were processed. PMFα, SMFα, and TMFα are the promoter, signal, and terminator sequences, respectively, of the gene for mating factor α (MFα) derived from *S. cerevisiae* (13).

Assay for glucoamylase activity. Glucoamylase activity was assayed by the method described previously (4). A 0.5-ml sample of an enzyme solution was incubated with 2.5 ml of a soluble starch solution and 1 ml of 10 mM McIlvaine buffer (pH 3.8) at 50°C for 10 min. The amount of reducing sugar formed was determined by the 3,5-dinitrosalicylic acid method (15); 1 U of glucoamylase activity was defined as the amount of enzyme that liberated 1 μmol of glucose per ml of reaction mixture per min under these conditions.

Binding to raw starch. A 1.0-ml sample of a solution of enzyme in 0.1 M citrate buffer (pH 4.0) was added to 200 mg of raw corn starch. The reaction mixture was incubated at 4°C for 30 min with occasional shaking. The glucoamylase activity of the supernatant was measured, and then the relative binding of the enzyme to raw starch was calculated.

Affinity chromatography on α-CD–Sepharose CL-4B. A 0.5-ml sample of a glucoamylase solution was loaded onto a column (0.64 cm [inside diameter] by 5 cm) of α-CD–Sepharose CL-4B (4) that had been equilibrated with 0.5 M NaCl in 0.1 M phosphate buffer (pH 5.5). The adsorbed glucoamylase was eluted with 2 mM β-CD in the same buffer.

Digestion of raw starch. The ability to digest raw starch was measured as described previously (4). Each reaction mixture, which contained 12.5 mg of raw corn starch and 2.0 ml of a solution of glucoamylase in 0.1 M citrate buffer (pH 3.6), was covered with 0.1 ml of toluene. The reaction mixtures were incubated at 30°C without shaking. At suitable times, the amount of reducing sugar formed was determined by the 3,5-dinitrosalicylic acid method, and the extent of hydrolysis was calculated.

Inhibition of digestion of raw starch by β-CD was also measured with the same reaction mixture containing 1 mM β-CD.

Analysis by difference spectroscopy of the reaction of GAW562L with β-CD. An analysis by difference spectroscopy of the reaction of GAW562L with β-CD was carried out by the method described previously (4).

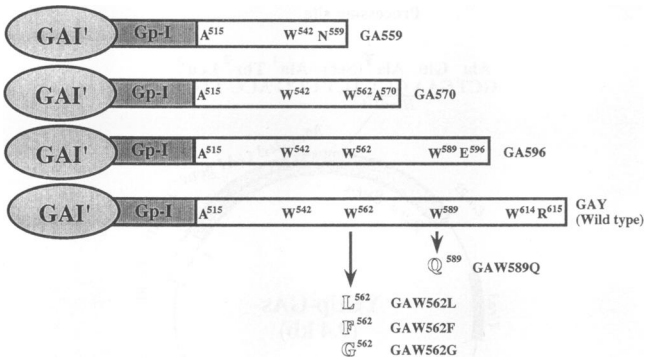

FIG. 2. Schematic representation of wild-type and mutant glucoamylases. Carboxy-terminally deleted glucoamylases and glucoamylases with point mutations were constructed by PCR and oligonucleotide-directed mutagenesis, respectively, as described in the text.

General analytical procedures. The molecular masses of glucoamylases were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using the method of Laemmli (14). Protein was quantified by determining the absorbance of solutions at 280 nm.

RESULTS

Design of mutant glucoamylases. We reported previously that one Trp residue in the Cp region of GAI contributes to the formation of inclusion complexes with cyclodextrins and raw starch (2, 4). As shown in Fig. 2, there are four Trp residues in the Cp region (7). Truncated glucoamylases, in which Trp residues were deleted one by one, were constructed to analyze the contribution of each Trp residue and the minimal region required for binding to and digestion of raw starch. Furthermore, as described previously (4), since it was suspected that Trp-562 might be involved in formation of the inclusion complex, Trp-562 was replaced by hydrophobic Leu-562 and Phe-562 residues, which should have been able to form inclusion complexes with cyclodextrins, and by Gly-562, which should not have been able to do so. Trp-589 was also replaced by hydrophilic Gln-589, which should not have been able to form inclusion complexes with cyclodextrins.

Properties of mutant glucoamylases. In many cases, heterologous expression of proteins in yeast cells results in hyperglycosylation of the proteins (12). Wild-type and mutant glucoamylases were also extensively glycosylated and produced broad bands on SDS-PAGE gels because of microheterogeneity due to glycosylation. As shown in Table 1, the molecular masses of wild-type GAY, GAW562L, GAW562F, GAW562G, and GAW589Q were estimated to be 115 kDa and the molecular masses of GA596, GA570, and GA559 were estimated to be 112, 110, and 110 kDa, respectively. The carbohydrate contents of these glucoamylases were higher than the carbohydrate content of *A. awamori* var. *kawachi* GAI (17%).

TABLE 1. Characteristics of various mutant glucoamylases[a]

Enzyme	Mol wt	Activity with soluble starch (U/mol)	Adsorbability onto raw starch (%)
GAY (wild type)	115,000	6.24×10^9	70.0
GA596	112,000	4.34×10^9	68.0
GA570	110,000	4.96×10^9	11.0
GA559	110,000	4.56×10^9	0
GAW562L	115,000	6.29×10^9	70.0
GAW562F	115,000	5.64×10^9	70.0
GAW562G	115,000	4.55×10^9	11.0
GAW589Q	115,000	5.92×10^9	70.0

[a] Experimental details are described in the text.

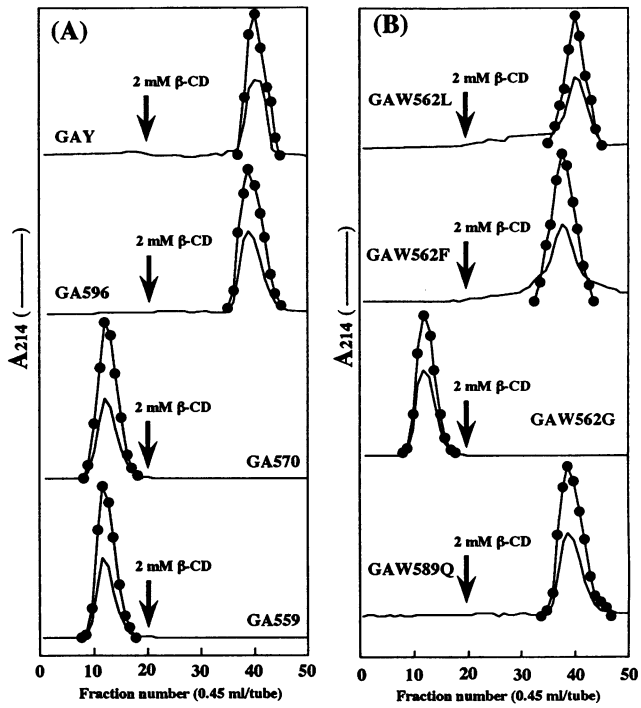

FIG. 3. Results of affinity chromatography of wild-type, carboxy-terminally deleted glucoamylases (A) and point-mutated glucoamylases (B) on α-CD–Sepharose CL-4B. The lines with no symbols indicate A_{214}. ●, glucoamylase activity. Experimental details are described in the text.

Hydrolysis of gelatinized starch by mutant glucoamylases. The catalytic activity of GAY was identical to that of GAI. Thus, an extra Ser residue at the amino terminus did not affect the catalytic activity. The ability of various mutant glucoamylases to hydrolyze gelatinized starch differed slightly from one enzyme to another, as shown in Table 1.

Binding of mutant glucoamylases to raw starch. As shown in Table 1, high levels of binding to raw starch (70%) were observed with GAY, GA596, GAW562L, GAW562F, and GAW589Q. However, the levels of binding of GA570, GA559, and GAW562G to raw starch were less than 12% under our experimental conditions.

Affinity of mutant glucoamylases for α-CD. As shown in Fig. 3A, the wild-type enzyme, GAY, adsorbed to α-CD–Sepharose CL-4B and was eluted with 2 mM β-CD with a peak at fraction 40. GA596 also adsorbed to this resin and was eluted with a peak at fraction 39. GA570 and GA559 did not adsorb to α-CD–Sepharose. However, as shown in Fig. 3B, GAW562L, GAW562F, and GAW589Q adsorbed to α-CD–Sepharose and were eluted by β-CD in the same peak fraction as wild-type GAY (fraction 40) and in earlier peak fractions (fractions 38 and 39), respectively. GAW562G did not adsorb to α-CD–Sepharose.

Digestion of raw starch by mutant glucoamylases. Since the catalytic activities of the various mutant glucoamylases against

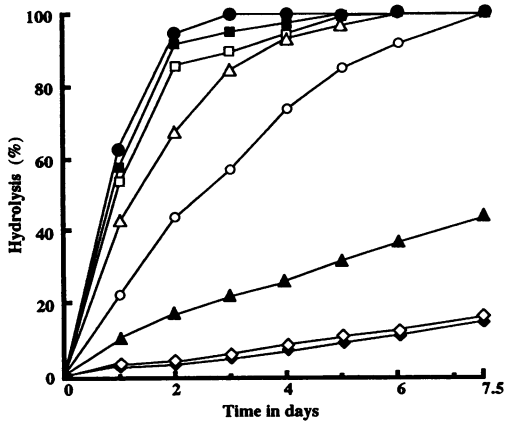

FIG. 4. Digestion of raw starch by wild-type and mutant glucoamylases. Symbols: ●, GAY; □, GA596; ○, GA570; ◇, GA559; ■, GAW562L; ▲, GAW562F; ◆, GAW562G; △, GAW589Q. Experimental details are described in the text.

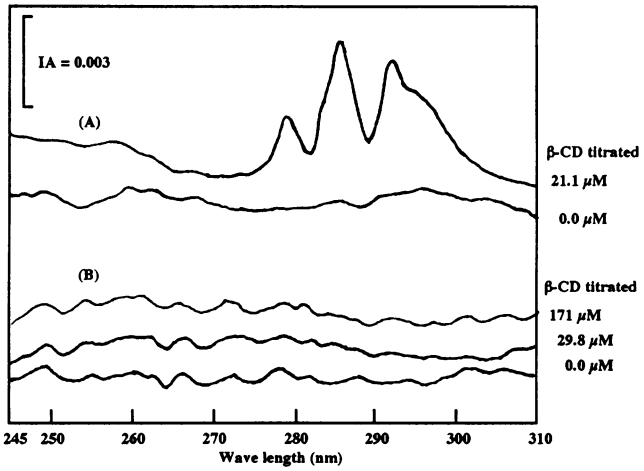

FIG. 6. Difference spectra in the UV region for GAI (A) and GAW562L (B) with and without β-CD. Experimental details are described in the text.

soluble starch differed from one another, digestion of raw starch by these enzymes was carried out under conditions in which the same level of catalytic activity was present in each reaction mixture (10 U/ml). As shown in Fig. 4, the ability of GA596 to digest raw starch was almost identical to the ability of the wild-type enzyme, GAY, while GA559 had lost this ability. However, GA570, which lacked two Trp residues, was able to digest raw starch despite its inability to bind to α-CD–Sepharose. In the case of mutant enzymes that had point mutations, GAW562L digested raw starch to the same extent as the wild-type enzyme, GAY. GAW562F exhibited significantly lower digestive ability (its ability was one-fifth the ability of GAY), and GAW562G was unable to digest raw starch. The ability of GAW589Q to digest raw starch was 70% of the ability of GAY.

Effect of β-CD on the digestion of raw starch by GA570. As shown in Fig. 5, digestion of raw starch by GA570 was specifically inhibited by β-CD.

Difference spectroscopy analysis of the reaction of GAW-562L with β-CD. As described previously (4), spectral perturbations that reflected the side chains of aromatic amino acids were observed during titration of GAI with β-CD. Although GAW562L adsorbed to β-CD–Sepharose, titration of GAW-562L with β-CD (0 to 220 μM) did not result in any spectral perturbations (Fig. 6).

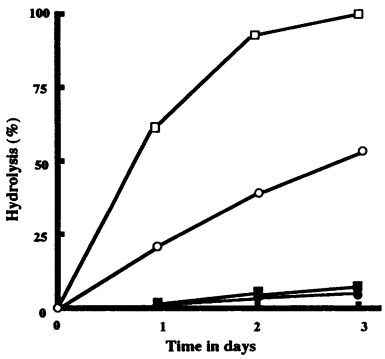

FIG. 5. Effects of β-CD (1 mM) on the digestion of raw starch by GA570 and GAY. Symbols: □, GAY; ■, GAY with β-CD; ○, GA570; ●, GA570 with β-CD. Experimental details are described in the text.

DISCUSSION

We suggested previously (4) that one Trp residue in particular, perhaps Trp-562, might contribute to the formation of an inclusion complex with a hydrophobic cavity in the amylose structure of raw starch via recognition of the secondary OH side of glucose residues.

In this study our data indicated that the minimal sequence around Trp-562, PL(W-562)YVTVTLPA, is essential for digestion of raw starch and that hydrophobic residue Trp-562 contributes to the formation of an inclusion complex. The sequence near Trp-589, which is rich both in amino acids that can form hydrogen bonds with the substrate and in charged amino acid residues, assists in the formation of the inclusion complex. The sequence from A-515 to N-559, including W-542, is not, by itself, sufficient for adsorption to and digestion of raw starch, but it may be necessary. The sequence from Y-597 to R-615, including W-614, is not important for the adsorption and digestion of raw starch (Fig. 7).

Our observation that GA596 adsorbs to and digests raw starch to a significant extent, while GA570 does not adsorb to α-CD–Sepharose and exhibits significantly lower affinity for raw starch but is able to digest raw starch, indicates that the sequence around Trp-562, PL(W-562)YVTVTLPA, is essential for the digestion of raw starch, while the sequence around Trp-589 is not essential for digestion but contributes to the adsorption to raw starch. Characteristics similar to those of GA570 were found in an α-amylase obtained from *Bacillus subtilis* 65 (10), which can digest raw starch but cannot adsorb to it.

Since GAW562L, GAW562F, and GAW589Q but not GAW562G bound extensively to raw starch and cyclodextrins, and since spectral changes during titration with β-CD were observed with GAI but not with GAW562L, it appears that Trp-562 forms an inclusion complex with the cavity of amylose and that newly introduced hydrophobic residues, such as Leu and Phe, can replace Trp in the interaction of the enzyme with its substrate.

The hydrophobic binding to raw starch, via formation of an

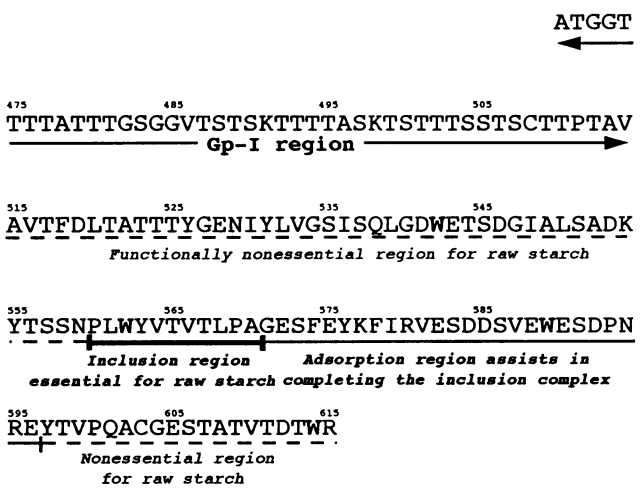

FIG. 7. Amino acid sequence and function of the Cp region (A-515 to R-615) as the raw starch-binding domain of *A. awamori* var. *kawachi* GAI. The inclusion region containing W-562 is essential for digestion of raw starch, and the adsorption region that is rich in charged and hydrogen bond-forming amino acids assists in completion of the formation of the inclusion complex. Both regions contribute to recognition of the structure of the secondary OH side of helical amylose.

inclusion complex between the cavity of the amylose and Trp-562 or equivalent hydrophobic residues, may cause the release from amylose of the included water to disrupt internal hydrogen bonds. The Gp-I region flanking the raw starch-binding domain interacts with a water cluster and supplies water molecules to the raw starch-binding domain, allowing GAI to digest raw starch effectively (3, 8).

REFERENCES

1. **Belshaw, N. J., and G. Williamson.** 1993. Specificity of the binding domain of glucoamylase 1. Eur. J. Biochem. **211:**717–724.
2. **Fukuda, K., Y. Teramoto, M. Goto, J. Sakamoto, S. Mitsuiki, and S. Hayashida.** 1992. Specific inhibition by cyclodextrins of raw starch digestion by fungal glucoamylase. Biosci. Biotechnol. Biochem. **56:**556–559.
3. **Fukuda, K., Y. Teramoto, and S. Hayashida.** 1992. The hyperdigestion of raw starch by a carbohydrate-rich glucoamylase from a protease- and glycosidase-negative mutant of *Aspergillus awamori* var. *kawachi* F-2035. Biosci. Biotechnol. Biochem. **56:**8–12.
4. **Goto, M., K. Tanigawa, W. Kanlayakrit, and S. Hayashida.** 1994. The mechanism of binding of glucoamylase I from *Aspergillus awamori* var. *kawachi* to cyclodextrins and raw starch. Biosci. Biotechnol. Biochem. **58:**49–54.
5. **Gunnarsson, A., B. Svensson, B. Nilsson, and S. Svensson.** 1984. Structural studies on the *O*-glycosidically linked carbohydrate chains of glucoamylase G1 from *Aspergillus niger*. Eur. J. Biochem. **145:**463–467.
6. **Hayashida, S.** 1975. Selective submerged production of three types of glucoamylases by black koji mold. Agric. Biol. Chem. **39:**2093–2099.
7. **Hayashida, S., K. Kuroda, K. Ohta, S. Kuhara, K. Fukuda, and Y. Sakaki.** 1989. Molecular cloning of the glucoamylase I gene of *Aspergillus awamori* var. *kawachi* for localization of the raw-starch-affinity site. Agric. Biol. Chem. **53:**923–929.
8. **Hayashida, S., K. Nakahara, W. Kanlayakrit, T. Hara, and Y. Teramoto.** 1989. Characteristics and function of raw-starch-affinity site on *Aspergillus awamori* var. *kawachi* glucoamylase I molecule. Agric. Biol. Chem. **53:**143–149.
9. **Hayashida, S., K. Nakahara, K. Kuroda, T. Miyata, and S. Iwanaga.** 1989. Structure of the raw-starch-affinity site on the *Aspergillus awamori* var. *kawachi* glucoamylase I molecule. Agric. Biol. Chem. **53:**135–141.
10. **Hayashida, S., Y. Teramoto, T. Inoue, and S. Mitsuiki.** 1990. Occurrence of an affinity site apart from the active site on the raw-starch-digesting but non-raw-starch-adsorbable *Bacillus subtilis* 65 α-amylase. Appl. Environ. Microbiol. **56:**2584–2586.
11. **Imberty, A., H. Chanzy, and S. Perez.** 1988. The double-helical nature of the crystalline part of A-starch. J. Mol. Biol. **201:**365–378.
12. **Innis, M. A., M. J. Holland, P. C. McCabe, G. E. Cole, V. P. Wittman, R. Tal, K. W. K. Watt, D. H. Gelfand, J. P. Holland, and J. H. Meade.** 1985. Expression, glycosylation, and secretion of an *Aspergillus* glucoamylase by *Saccharomyces cerevisiae*. Science **228:**21–26.
13. **Kitada, K., and F. Hishinuma.** 1988. Evidence for preferential multiplication of internal unit in tandem repeats of the mating factor α genes in *Saccharomyces* yeasts. Curr. Genet. **13:**1–5.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Miller, G. L.** 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. **31:**426–428.