Evidence for Post-translational Generation of Multiple Forms of Aspergillus awamori var. kawachi Glucoamylase

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Aspergillus awamori var. kawachi glucoamylase (GA) existed in three molecular forms varying in size, raw starch digestion and hydrolysis curve toward glycogen. The largest form, glucoamylase I (GAI, MW 90,000), adsorbed onto raw starch and digested it, while the other two forms, GA' (MW 83,000) and GAII (MW 57,000), acted on gelatinized starch and glycogen. The in vitro treatment of raw starch-digesting GAI with fungal acid protease or subtilisin resulted in formation of raw starch-nondigesting and -unadsorbable GA' by liberating a glycopeptide I (Gp-I, MW 7,000). Since the Gp-I region on the GAI molecule was distant from the active site and essential for the adsorption onto raw starch in the digestion, the Gp-I was designated as the "raw starch-affinity site". Similarly, the raw starch digestion of Rhizomucor pusillus glucoamylase was correlated to the presence of a raw starch-affinity site. On the other hand, Boel et al. proposed that A. niger produced two forms (Gl) and (G2) of glucoamylase via splicing of mRNA coding for the larger form, G1. However, since the structure of the smaller form G2 differed from the presumptive G2 mRNA in the C-terminal sequence, it was suggested that G2 was generated by post-translational proteolysis of G1. For this paper, the amino acid sequences of both N- and C-terminals of Gp-I were analyzed and the location of the GAI molecule was assigned by comparing with the complete amino acid sequence of A. awamori GA1 determined by Nunberg et al. Moreover, in vitro translation of A. awamori var. kawachi mRNA and immunoprecipitation with GAI-specific antisera were described to allow discussion of the mechanism for generation of multiple forms of the fungal glucoamylase.

A. awamori var. kawachi was grown in synthetic medium A (120 l) in a 200-l stainless steel fermentor (B.E. Marubishi Co., Ltd.) with agitation at 250 rpm and aeration at a rate of 1vvm at 32°C for 36 hr. GAI produced was purified according to the method previously described. Gp-I was prepared by cleaving GAI by subtilisin. The crude preparation of Gp-I was subjected to reversed-phase high-performance liquid chromatography on a Finepak SIL C18 column (250 × 4.6 mm). The column was eluted with a 30-min linear gradient of 0–50% acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow rate of 0.5 ml/min at room temperature. Purified Gp-I was denatured with 6 M guanidine hydrochloride in 0.5 M Tris-HCl buffer (pH 8.25) containing 20 mM EDTA, reduced with dithiothreitol, and treated with 4-vinylpyridine. The S-pyrrolidylated Gp-I was sequenced using an Applied Biosystems gas-phase sequencer (model 470A) and an on-line phenylthiohydantoin amino acid analyzer (model 20A). The C-terminal amino acid of Gp-I was identified using carboxypeptidase Y and W. For its amino acid composition, Gp-I was hydrolyzed with 6 N HCl at 110°C for 24 hr and the amino acid composition of the residues was determined by the procedure of Spackman et al. with a Hitachi amino acid analyzer (model 835).

Purified GAI protein, emulsified with Freund's complete adjuvant, was injected into rabbits to generate the antisera. GAI-specific antisera were purified by affinity chromatography. Total RNA was isolated from A. awamori var. kawachi mycelia by using the guanidinium thiocyanate procedure. Mycelia were wrenched dry in cheesecloth and ground to a powder in liquid nitrogen with a mortar and pestle. The powder was suspended in a buffer (pH 7.0) containing 4 M guanidinium thiocyanate, 0.5% sodium N-lauroylsarcosine, 25 mM sodium citrate, and 100 mM 2-mercaptoethanol. The suspension was homogenized in the presence of quartz sand (20 ~ 30 mesh). After centrifugation to pellet cell debris at 10,000 × g for 10 min, RNA was isolated from the supernatant by pelleting through a CsCl pad in an SW41 Beckman rotor run at 35,000 rpm for 15 hr at 25°C as described by Glassin et al. Polyadenylated RNA was isolated by two cycles of oligodeoxynucleotidylate cellulose chromatography and translated in vitro by the rabbit reticulocyte lysate system (Amersham) with [4,5-3H]leucine as described by the supplier. Purified rabbit GAI-specific antisera were added to the lysates and immune complexes were precipitated by Formalin-fixed Staphylococcus aureus Cowan cells.

The amino acid composition of Gp-I was abundant in hydroxyamino acids such as threonine and serine (Fig. 1A), which correlated with the high carbohydrate content in Gp-I. The N- and C-terminal amino acids were alanine and valine, respectively. Both the third and fourth amino acids from the N-terminal were glycine. Threonine and serine could not be detected, presumably due to O-glycosidic linkages of carbohydrate to the hydroxy groups.
Fig. 1. (A) N- and C-terminal Amino Acid Sequences of *Aspergillus awamori* var. kawachi Glycopeptide I and (B) Amino Acid Sequence of *Aspergillus awamori* Glucoamylase I (Nunberg et al.9). The underline represents the proposed region of glycopeptide I on the glucoamylase I molecule. The arrow indicates the peptidyl bonds cleaved by subtilisin.

The comparison of partial N- and C-terminal amino acid sequences of Gp-I with the complete amino acid sequence of *A. awamori* GAI determined by Nunberg et al.9) suggested the location of Gp-I on GAI molecule between Ala471 and Val515 of 616 amino acid residues (Fig. 1B). It also implied that subtilisin cleaved *A. awamori* var. kawachi GAI at the two peptidyl bonds of Val470-Ala471 and Val515-Ala516.

The products of *in vitro* translation of mRNA and immunoprecipitation were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). One protein with an apparent molecular weight of 69,000 was precipitated with the antisera as the most abundant translation product. The molecular weight of the major product was consistent with that estimated for an unglycosylated form of GAI. For this study, GAI was converted to GAr by liberating Gp-I at the above two peptidyl bonds by *in vitro* treatment with subtilisin. Unlike the *A. niger* G2, the resultant GAI consisted of two polypeptide chains.2) GAIII, a single polypeptide chain, was generated from GAI or GAF by the cooperative action of glycosidases and protease.2,3) The proposal of post-translational generation of multiple forms of *A. awamori* var. kawachi glucoamylase was also supported by the *in vitro* translation experiment (Fig. 2) showing that one major glucoamylase polypeptide was synthesized. The complete amino acid sequencing of Gp-I and GAI is in progress.

REFERENCES


