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MOLECULAR WEIGHT OF THE UNDEGRADED POLYPEPTIDE CHAIN OF *PSEUDOMONAS AMYLODERAMOSA* ISOAMYLASE

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Summary

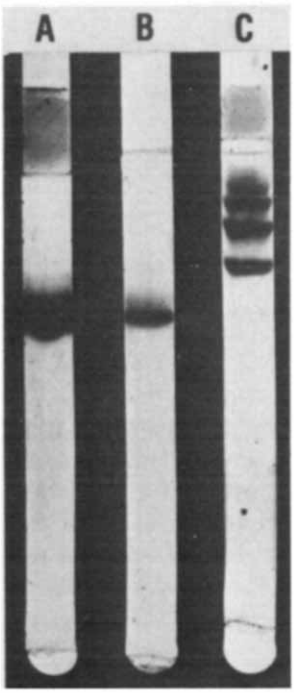
Crystalline isoamylase of *Pseudomonas amyloderamosa* was found to be contaminated with a trace of proteolytic enzyme. This contaminant digested the isoamylase under neutral or alkaline conditions, especially in the presence of sodium dodecyl sulfate (SDS). A reliable molecular weight of the enzyme was obtained by SDS-polyacrylamide gel electrophoresis and by gel filtration on Sepharose-6B in 6 M guanidine-hydrochloride after heat inactivation of the contaminant. The molecular weight of the undergraded polypeptide chain of the isoamylase was about 90 000. The lower molecular weight and the subunit structure of the enzyme reported previously are incorrect.

Pseudomonas isoamylase [1—5] (glycogen 6-glucanohydrolase, EC 3.2.1.68) cleaves β-(1→6)-D-glucosidic linkages in amylopectin and glycogen. The enzyme was isolated by Harada et al. [1] from *Pseudomonas amyloderamosa*. Subsequently, we studied the purification [2, 6] and properties [2, 7, 8] of the enzyme and the structures [3, 4] of amylopectin and glycogen by debranching them with the enzyme.

The molecular weight of the native enzyme was estimated as 94 000 by centrifugation analysis [2], and that of the subunits as 46 000 by sedimentation equilibrium analysis in the presence of 6 M guanidine hydrochloride [7], and as 52 000 by gel filtration on Sepharose 6B in the presence of 5 M guanidine hydrochloride [7]. However, later we found that the subunit structure of this enzyme was dubious. This paper reports that the crystalline isoamylase was contaminated with a trace of proteolytic enzyme and that the apparent subunit structure was due to this contaminant.

Crystalline isoamylase of *Ps. amyloderamosa* strain KlC was prepared from material purified by affinity chromatography [6] on cross-linked amylose gel. Before use, the enzyme was stored as a suspension of crystals in ammonium sulfate in 0.01 M acetate buffer (pH 4) at 4°C. When the crystalline isoamylase was subjected to polyacrylamide gel electrophoresis (pH 8.3) at 4°C, it gave a single band (Fig. 1A and 1B). But when the electrophoresis was run at room temperature, several bands of lower mobility appeared (Fig. 1C), suggesting some proteolysis under these conditions.

When the enzyme preparation was subjected to SDS-polyacrylamide gel electrophoresis after treatment with 1% SDS and 1% 2-mercaptoethanol for 1 h at 40°C many distinct bands appeared with molecular weights of 80 000—15 500 (Fig. 2A). Longer treatment with SDS (24 h) resulted in fragmentation of the protein with smaller peptides with molecular weights of 25 000—8 000 (Fig. 2B). This proteolysis was reduced by heating the sample for 4 min immediately after adding the SDS and 2-mercaptoethanol (Fig. 3A). This treatment inactivated the contaminating protease before it could digest the isoamylase.



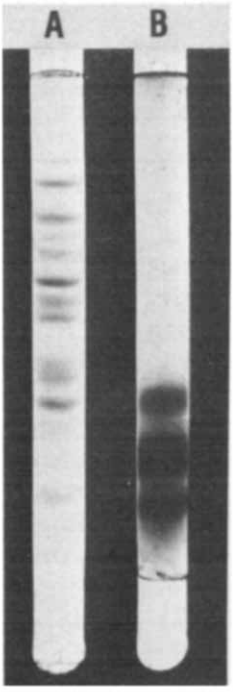


Fig. 1. Polyacrylamide gel electrophoresis of isoamylase at pH 8.3. Gels were run by the method of Davis [9]. Approx. 0.2 mg (A, C) or 0.05 mg (B) of protein was applied to the gel, and run at 4°C (A, B) or at room temperature (C) for 2 h with a current of 4 mA/gel. After the run, the gels were stained with Amido Black 10B.

Fig. 2. Polyacrylamide gel electrophoresis of isoamylase in the presence of SDS. Gels were run by the method of Weber and Osborn [10]. The sample was dissolved in 0.01 M phosphate buffer (pH 7.2) containing 1% SDS and 1% 2-mercaptoethanol and incubated for 1 h (A) or 24 h (B) at 40°C. After incubation, the mixture was applied to gel containing 0.1% SDS in 0.1 M phosphate buffer (pH 7.2), and run for 4 h at room temperature with a current of 8 mA/tube. Then the gels were stained with Amido Black 10B.

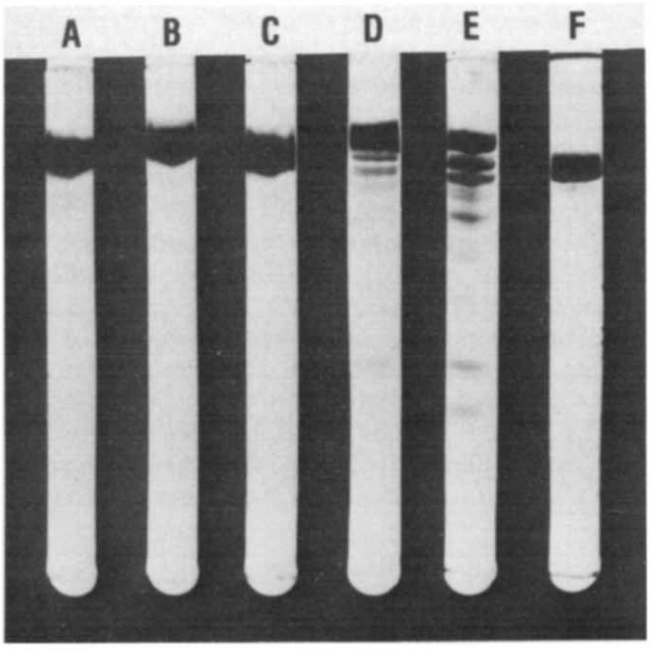


Fig. 3. Polyacrylamide gel electrophoresis of isoamylase in the presence of SDS. The sample was incubated for 2 h in 0.05 M chloride buffer, pH 2 (B), 0.05 M acetate buffer, pH 5 (C), 0.05 M phosphate buffer, pH 7.2 (D) or 0.05 M phosphate buffer, pH 8.3 (E), diluted with a solution of 1% SDS and 1% 2-mercaptoethanol in 0.01 M phosphate buffer (pH 7.2), heated for 4 min at 100°C, then applied to the gel with SDS. A, without incubation in buffer; F, heated before incubation in phosphate buffer (pH 8.3).

Proteolysis also occurred to some extent when the sample was incubated under neutral or alkaline conditions without SDS and 2-mercaptoethanol. For instance, as shown in Fig. 3D and E, several bands of smaller molecular weight material were detected when the sample was incubated at pH 7.2 or 8.3 in 0.05 M phosphate buffer for 2 h at 40°C, heated for 4 min at 100°C immediately after adding 1% SDS and 1% 2-mercaptoethanol and then subjected to SDS-polyacrylamide gel electrophoresis. Incubation at pH 2 in 0.05 M chloride buffer or at pH 5 in 0.05 M acetate buffer (Fig. 3A and B) and incubation at pH 8.3 after heating the enzyme for 4 min at 100°C at pH 4 (Fig. 3F) did not cause any fragmentation.

The molecular weight of the undegraded polypeptide chain was estimated at 86 000 by SDS-polyacrylamide gel electrophoresis by comparison with standard proteins.

Gel filtration of the enzyme on Sepharose-6B in guanidine hydrochloride was reinvestigated. The sample was first heated for 4 min at 100°C to inactivate protease, and then reduced with 0.1 M dithiothreitol, alkylated with monoiodoacetic acid in 1 M Tris-HCl (pH 8.5)/6.5 M guanidine hydrochloride solution, and applied to a column of Sepharose-6B in 6 M guanidine hydrochloride. The molecular weight of isoamylase calculated by comparison of the elution volume of isoamylase with those of standard proteins was 88 000. Previous results [7] indicating a molecular weight of 52 000 were probably obtained because of partial proteolytic degradation of the enzyme

in Tris-HCl (pH 7.6)/guanidine hydrochloride solution at room temperature.

The molecular weight of 94 000, obtained previously for the native isoamylase [2] under conditions (pH 4) in which the protease does not act on isoamylase, seems reliable. From comparison of this molecular weight with those of 86 000 and 88 000 obtained for the enzyme with protein denaturants, it is concluded that isoamylase of *Ps. amyloderamosa* has no subunits.

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