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Note

Simple Affinity Purification Method for Raw Starch-adsorbable and -digesting Amylases with a Raw Starch Column

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A simple purification procedure for raw starch-adsorbable and -digesting amylases (RSAs) was devised. The method depended on an affinity column, which was prepared by mixing raw corn starch and Hyflo Super-Cel. RSAs were specifically adsorbed on the matrix, and eluted with a buffer containing 1% β-cyclodextrin. This column could be used to purify RSAs from *Streptomyces thermocyaneoviolaceus* and a recombinant strain of *E. coli*.

Key words: amylase; raw starch; affinity chromatography; raw starch-digesting amylase

Raw starch-digesting amylases (RSAs) are found in various kinds of microorganisms, including bacteria, fungi, and actinomycetes.[1] Enzymatic properties of many RSAs have been examined, and successful use of the enzyme for ethanol fermentation also have been reported.[2] Recent detailed studies on RSA from *A. awamori* var. *kawachii* demonstrated that the enzyme has two domains and an essential Trp residue for formation of inclusion complexes between the enzyme and the substrate, raw starch.[3] One might expect further analysis of the reaction mechanism of the enzyme using mutant enzymes designed by site-directed mutagenesis, and comparative studies on the enzymes from organisms that produce only low levels of the enzyme.

For such studies, we need a simple purification method for RSAs, the cost of which should be as low as possible. Affinity chromatography with CD-Sepharoses has been used for purification of RSAs. However, this useful method suffers some disadvantages: the high price of the matrixes, difficulty of usage for application of crude enzyme samples, and requirement of different types (α-, β-, and γ-) of CD-Sepharose, depending on the affinity of the enzyme to be purified toward CD.[4] If we could use the raw starch as an affinity matrix, these problems will be overcome. Interestingly, in spite of many studies on RSAs, as far as we know, no successful study has been reported on affinity chromatography with raw starch.

This report describes a procedure of simple affinity chromatography using raw starch and Hyflo Super-Cell, which was successfully used for purification of the RSAs from *Streptomyces thermocyaneoviolaceus* IFO 14271 and a recombinant strain of *E. coli*.

The soluble starch-digesting activity was measured at 40°C by the Nelson–Somogyi[5] reducing sugar assay with a reaction mixture consisted of the enzyme, 10 mM sodium acetate buffer (pH 6.0), and 1% soluble starch. One unit of the enzyme activity was defined as the amount of the enzyme that liberated the reducing equivalent of 1 μmol of D-glucose per minute. The raw starch-digesting activity was measured with a reaction mixture (2 ml) consisted of the enzyme, 10 mM sodium acetate buffer (pH 6.0), and 1% raw corn starch. The reaction mixture was incubated at 40°C for 20 min with shaking (160 strokes/min). After removal of granules, total sugar in the supernatant solution was measured by the method of Dubois *et al.*[6] with glucose as a standard. One unit of the raw starch-digesting activity was defined as the amount of the enzyme that produces 1 μmol of total sugar per min. Protein concentration was measured by the method of Lowry *et al.*[7] with bovine serum albumin as a standard. The purity of the enzyme preparations were checked by SDS–PAGE by the method of Laemmli.[8]

S. thermocyaneoviolaceus (IFO 14271) were reciprocally cultivated at 46°C for 72 h with a medium composed of 3.0% corn starch, 0.5% Bacto-soytone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.05% $CaCl_2 \cdot 2H_2O$, 0.05% $MgCl_2 \cdot 6H_2O$, and 0.05% $MnCl_2 \cdot 4H_2O$. A recombinant strain of *E. coli, JM 109*, which contained a plasmid carrying the raw starch-digesting amylase gene from *Zoogloea ramigera* KO 159, was cultured in the LB medium (2 liters) at 30°C for 24 h. The preparation method for the recombinant strain will be described elsewhere.

We have examined raw starches of wheat, sweet potato, and corn, which were kindly supplied from Amano Pharm. Co., Ltd., for using as the matrix. The flow rates of columns made with one of these starches only were too slow to use the columns for chromatography. Thus, Hyflo Super-Cel, a filtration aid purchased from Wako Chemical Industries, Osaka, was mixed with the starches in several mixing proportions. The higher proportions of Hyflo Super-Cel gave the higher flow rates and the lower potential of adsorption of RSA. The mixture of 4 parts of starch and 1 part of Hyflo Super-Cel gave the best result in view of these two factors. Corn starch was the best among the raw starches examined: although RSA from *S. thermocyaneoviolaceus* showed 1.5 fold higher activity toward raw wheat starch than toward corn starch, the latter showed a higher potential of adsorption of RSA than the former. Then, a working affinity column was prepared as follows. Raw corn starch (160 g) and 40 g of Hyflo Super-Cel were thoroughly mixed in 500 ml of water. This was left at room temperature for about 1 h. The supernatant was discarded by decantation. The sediment, which was resuspended in 400 ml of 0.01 M sodium acetate buffer (pH 6.0), was poured into a column. The final volume of the column was 269 ml (3.5 × 28 cm). The column was equilibrated with 200 ml of Buffer A (0.01 M sodium acetate buffer, pH 6.0, containing 0.15 M NaCl).

The culture broth (850 ml) of *S. thermocyaneoviolaceus* was centrifuged, and the supernatant obtained was fractionated by 80% saturation of ammonium sulfate. The precipitated enzyme fraction was dissolved in a limited amount of Buffer A and then the solution was dialyzed thoroughly against Buffer A. The dialyzed solution was put on the column described above. Figure 1A shows the elution pattern of the column chromatography. Almost all proteins were eluted with Buffer A and Buffer B (0.01 M sodium acetate buffer, pH 6.0, containing 1.0 M NaCl). RSA was specifically eluted with Buffer B containing 1% of β-CD and

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Abbreviations: RSA, raw starch-digesting amylase; CD, cyclodextrin.

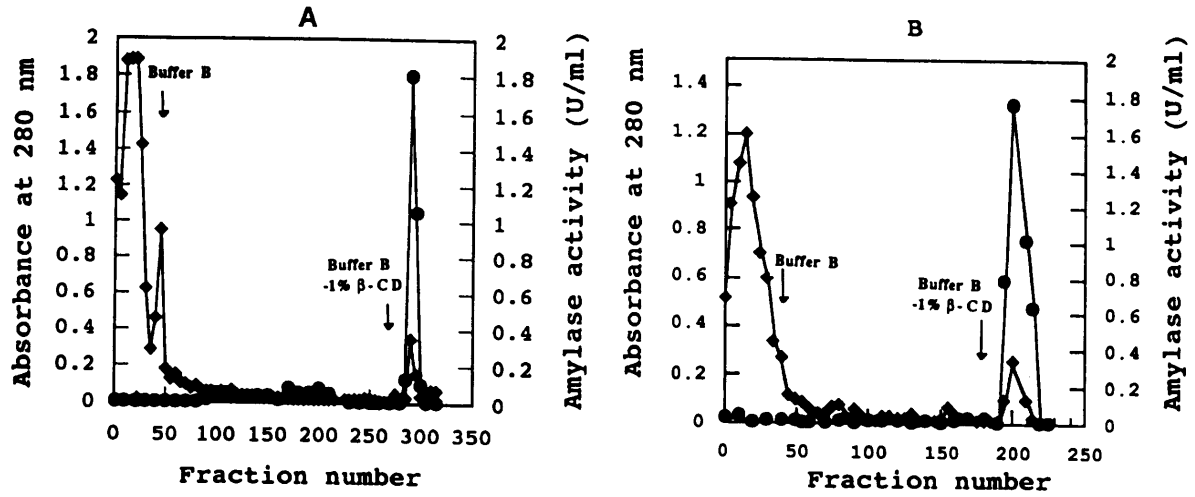

Fig. 1. Elution Pattern of Raw Starch-digesting Amylases of *S. thermocyaneoviolaceus* (A) and Recombinant Strain of *E. coli* (B).

A: The crude enzyme solution was put on the affinity column (3.5 × 28 cm), and then the column was washed with 300 ml of Buffer A, 3 liters of Buffer B, and 500 ml of Buffer B containing 1% β-CD and 0.02% sodium azide. B: The column was washed with 450 ml of Buffer A, 1.8 liters of Buffer B, and 500 ml of Buffer B containing 1% β-CD and 0.02% sodium azide. Absorbance at 280 nm (◆) and the enzyme activity (●) of each fraction were measured after washing out β-cyclodextrin from the solutions by ultrafiltration with Millipore Ultrafree CL (UFC 4 LCG 25). The pumping speed of the solutions was about 30 ml/h. Each fraction tube contained 15 ml of the solution. The column chromatography was done at 4°C.

Table Comparison of Purification Methods of Raw Starch-digesting Amylase from *S. thermocyaneoviolaceus* IFO 14271

Method	Step	Total activity (R-Activity)	Total protein (mg)	Specific activity (U/mg)		Yield (%)
				R-Activity	S-Activity	
Raw starch affinity column	Culture filtrate	349	2120	0.17	0.8	100
	Ammonium sulfate precipitation	261	277	0.9	4.3	75
	Raw corn starch affinity column	105	3.7	28	135	30
	FPLC MONO Q HR 5/5	92	2.8	33	158	26
Conventional method	Culture filtrate	188	1050	0.18	0.9	100
	Ammonium sulfate precipitation	144	150	1	4.0	76
	Raw corn starch treatment	86	15	6	29	46
	Utrogel AcA34 (1)	61	4	15	73	33
	FPLC MONO Q HR 5/5	56	3	19	90	31
	Phenyl-Sepharose	45	1.7	27	129	24
	Ultrogel AcA34 (2)	40	1.4	29	138	21

R-Activity and S-activity indicate raw starch-digesting and soluble starch-digesting activity, respectively.

0.02% sodium azide. β-CD was used because it is well known that CDs interact with the raw starch binding domain of RSAs and inhibit binding of RSAs to raw starch,[9] and β-CD was the lowest price among commercially available CDs. The RSA fraction obtained was put on the SDS–PAGE as shown in Fig. 2. The RSA fraction gave a major protein band with molecular weight of 49,000 and a minor band, which can hardly be seen on the picture. The RSA was purified 169-fold by the affinity chromatography, and the specific activity of the RSA became 135 (unit/mg). Final yield of the purification procedure was 26%. The purification steps are summarized in the Table. The purification of RSA from the culture filtrate of *S. thermocyaneoviolaceus* by the conventional method is shown in the Table to compare with the affinity method. Description of details of the conventional method, such as conditions of various column chromatographies were omitted because it is beyond the scope of this paper.

The crude extract (50 ml) of the recombinant strain of *E. coli* was centrifuged to obtain a supernatant solution. The supernatant solution was put on the affinity column. Figure 1B shows RSA in the recombinant strain was also purified effectively with the column. The RSA obtained was homogeneous as shown in Fig. 2. The molecular weight of the enzyme was 63,000. The RSA was

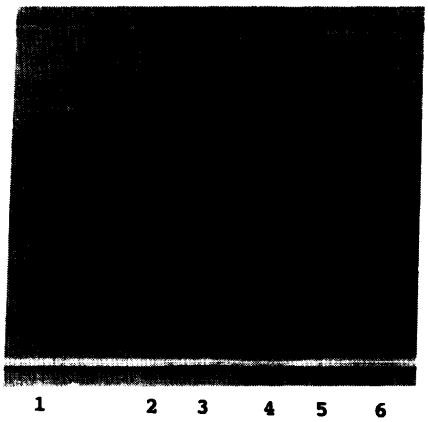

Fig. 2. SDS–PAGE Patterns of Fractions Obtained during Purification of Raw Starch-digesting Amylases.

The affinity-purified enzyme solution (35 μg of protein), ammonium sulfate-fractionated enzyme solution (360 μg), and the crude enzyme solution (240 μg) from *S. thermocyaneoviolaceus* were put on lanes 1, 2, and 3, respectively. To lanes 5 and 6 put on the crude enzyme solution (215 μg) and the affinity-purified enzyme solution (35 μg) from the recombinant strain, respectively. Lane 4 shows standard proteins: rabbit muscle phosphorylase (M_r 97,400); bovine serum albumin (66,200); hen egg white albumin (45,000); bovine carbonic anhydrase (31,500); soy bean trypsin inhibitor (21,500); hen egg white lysozyme (14,400).

purified 23-fold, and the specific activity of the RSA fraction was 62.2. The final yield of the purification procedure was 38.3%.

The raw starch-Hyflo Super-Cel column was very effective for purification of RSAs. So far, many workers have used raw starches to specifically precipitate and purify RSAs as described in the conventional method. However, effectiveness of the specific precipitation procedure is generally moderate, and several other purification steps such as an ion-exchange column chromatography are required to purify RSAs to homogeneity.[10] Thus, making a column and specific elution with β-CD may be essential to get good efficiency of RSA purification with raw starches.

This column was not reusable. RSAs in second samples did not adsorb onto the column matrix once it was used, even after the columns were washed thoroughly with Buffer B and then Buffer A. Reactivation conditions of this column are currently unknown.

The raw starch-Hyflo Super-Cel column may be applicable for all enzymes and proteins that have affinity toward raw starch, including RSAs. Furthermore, this column may be useful for identification of essential residues for adsorption of RSAs to starch if mutant enzymes, which will be made by site-directed mutagenesis, were used in combination with this column.

S. thermocyaneoviolaceus and *Z. ramigera* seemed to produce only the one type of RSA described here: we could not detect the other RSA activities during purification of the RSAs from the bacteria. Enzymatic properties of amylase from *Z. ramigera* have been presented previously.[11] Enzymatic properties of RSA from *S. thermocyaneoviolaceus* will be described elsewhere. Molecular weight of the RSAs have been reported to be 68,000–93,000. Thus, the RSA from *S. thermocyaneoviolaceus* showed a characteristically low molecular weight.

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