J. Microbiol. Biotechnol. (2003), 13(2), 313–316

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Demonstration of Two Independent Dextranase and Amylase Active Sites on a Single Enzyme Elaborated by *Lipomyces starkeyi* KSM 22

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Received: September 16, 2002
Accepted: November 28, 2002

Abstract *Lipomyces starkeyi* KSM 22 elaborates an enzyme that has both dextranase and amylase activities in a single protein of 100 kDa. Competition studies, using different amounts of dextran and starch as substrates, gave a competition plot consistent with the hypothesis that the hydrolysis of dextran and starch occurs at two independent active sites, each specific for starch and dextran, respectively.

Key words: Dextranase, amylase, *Lipomyces starkeyi*, active site, competition plot

Endodextranase (EC 3.2.1.11) catalyzes the hydrolysis of contiguous α-(1→6) glucosidic linkages of dextran, and endoamylase (EC 3.2.1.1) cleaves the α-(1→4) glucopyranosyl linkages in starch. Culture supernatants of a constitutive mutant of *Lipomyces starkeyi* ATCC 74054 had both endo-dextranase and endo-amylase activities [12]. Kim *et al.* [14] obtained a hyperproducing dextranase and amylase mutant, *L. starkeyi* KSM 22, by chemical mutagenesis using ethyl methane sulfonate. They purified both dextranase and amylase activities, and found that both activities resided in a single protein, having a molecular mass of 100 kDa, determined by both denaturing and nondenaturing SDS-PAGE [17, 19, 24]. This enzyme was given the abbreviation of DXAMase for dextranase-amylase. When DXAMase was produced by the mutant in a starch medium, it hydrolyzed the insoluble *Streptococcus mutans* glucan 3.75-fold more than *Penicillum funiculosum* dextranase did.

Aggregation of *S. mutans* cells with dextran and their adherence to glass vials were eliminated by incubating with DXAMase. The addition of DXAMase to a reaction mixture of mutansucrase with sucrose reduced the formation of insoluble glucan by 80%. DXAMase also removed 80% of the preformed sucrose-dependent adherent film. These properties of *L. starkeyi* KSM 22 DXAMase have potential use as an agent in the control and/or elimination of dental plaque. The details of the catalytic action of DXAMase have not yet been elucidated. In this study, the kinetics of DXAMase for the hydrolysis of a mixture of dextran and starch were examined to determine whether DXAMase has the same active site for the hydrolysis of dextran and starch or two separate active sites, one for each of two substrates.

L. starkeyi KSM 22 was cultured in LW medium [0.3% (w/v) yeast extract, 0.3% (w/v) KH_2PO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001% (w/v) NaCl, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.001% (w/v) $MnSO_4 \cdot H_2O$, and 0.013% (w/v) $CaCl_2 \cdot 2H_2O$] containing 1% (w/v) soluble starch in a 10-l jar-fermenter (BioTron Co., Korea) for the production of DXAMase [11, 13, 14–15, 22, 28–29]. After the fermentation was complete (60 h), the cells were removed by centrifugation and the pH was adjusted to 4.5 with 1 M HCl. The protein concentration was determined by the Bradford method [2], and the enzyme activities were determined by the copper-bicinchoninate method using 1% substrate [5]. To assay the dextranase and amylase activities, B-512F dextran (10 mg/ml) and soluble starch (10 mg/ml) were used at pH 5.5, respectively [16, 18, 21]. An International Unit (IU) of enzyme activity was defined as one μmole of glucosidic bonds cleaved/min at 37°C and pH 5.5, determined using maltose as a standard in the reducing value assay. The culture supernatant had 2.2 IU/mg dextranase and 1.8 IU/mg amylase activities. It was concentrated from 7.5 l to 610 ml, using a 30,000 MW cutoff hollow-fiber membrane (Millipore, Beverly, MA, U.S.A.). The concentrated enzyme had 4.6 IU/mg dextranase activity and 2.4 IU/mg amylase activity. The enzyme was further concentrated and purified by slowly adding 80% of ammonium sulfate to 610 ml.

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The resulting precipitate was dissolved in 50 ml of citrate phosphate buffer (pH 5.5) and dialyzed against 5 l of citrate phosphate buffer (pH 5.5) to give 60 ml of enzyme, having 10.4 IU/mg dextranase activity and 2.6 IU/mg amylase activity. DEAE-Sepharose (Sigma Chemical Co., St Louis, MO, U.S.A.) column (38 cm × 1.8 cm) was prepared and equilibrated with 20 mM potassium phosphate buffer (pH 6.4). The crude concentrate was applied to the column and the enzyme was eluted with 0- 1.5 M NaCl linear gradient, and 7-ml fractions were collected. The active fractions were pooled and applied to the DEAE-Sepharose column for the second time, which was repeated two more times, resulting in a final volume of 45 ml with 366.4 IU/mg dextranase activity and 161 IU/mg amylase activity.

Enzymes often act on more than one substrate and sometimes, with substrates with considerable differences in structure, so that two different kinds of enzyme activities seem to reside in a single enzyme [9, 27]. A question then arises as to whether these activities can be attributed to the existence of two different enzymes that have not been separated or possibly to the presence of two different active sites in the same enzyme. In the latter situation, a kinetic competition experiment, using the two different substrates in different amounts, can be used to determine whether or not the two enzyme activities are catalyzed at the same active site or at two independent active sites [3]. The experiment consists of making mixtures of the two substrates and plotting the total rate of the different mixtures against a parameter p that defines the concentrations of the two substrates in terms of reference concentrations chosen to give the same rates. That is, at p=0, concentration of substrate A gives a rate, r_a, and at p=1, a concentration of substrate B gives a rate, r_b that is equal to r_a. Then different amounts of substrates A and B are used to give values (1-p) between 0 and 1. Chevillard *et al.* [4] give the details of the theoretical aspects of the resulting competition plot.

The competition plot was obtained by making dextran, substrate A, and starch, substrate B. A reference concentration, a_o, for dextran was chosen and its rate of hydrolysis, v_a, was determined. The concentration of starch was determined that gave a rate, v_b, that was equal to v_a. The a_o concentration of dextran was 1.4 mg/ml and gave a v_a of 2.7 mmoles of maltose/min/ml. It was then determined that the concentration of b_o of starch was 10 mg/ml to give a v_b of 2.7 μmoles of maltose/min/ml, equal to the v_a obtained for dextran. A reaction volume of 500 μl was used to determine the initial velocities for each substrate concentration. Digests were prepared, keeping the amount of enzyme and other components of the reaction fixed and mixing solutions of A (dextran) and B (starch) so that a equals 1.4 (1-p) mg/ml dextran and b equals 10 p mg/ml starch. A dextran solution (2 mg/ml) and a starch solution (12 mg/ml) were mixed in varying proportions, according to the following formuler: {1.4 (1-p)}/2×450 μl of dextran solution, containing 0.1 M citrate-phosphate buffer (pH 5.5) and {(10 p)/12}× 450 μl of starch solution, containing 0.1 M citrate-phosphate buffer (pH 5.5) which resulted in a solution of 450 μl of the two substrates and 0.1 M citrate buffer (pH 5.5). The substrates were incubated at 37°C and the reaction was initiated by adding 50 μl (0.135U) of DXAMase. Aliquots were taken at various times and the reducing-value (μmoles of maltose equivalent/ml) was determined. The reducing value was plotted *vs* time and the slope of the linear line was the v_o for each mixture of substrates. A competition plot of v_o vs p was then obtained.

If the two substrates react at the same active-site, the competition plot gives a horizontal straight line and the rates are independent of p. If reactions of the two substrates are each at a different and independent active site, a curve with a maximum occurs. If there are two active sites, and A is an inhibitor for the active site of B and B is an inhibitor for the active-site of A, the height of the maximum decreases. It may even be converted into a minimum, if the substrate is more effective as an inhibitor for the active-site of the other substrate than it is a substrate for its own active site [1, 20, 25- 26].

The results of the competition plot for DXAMase (Fig. 1) show that the mixtures of dextran and starch were degraded faster than the degradation of the two individual substrates. These results indicate that the reaction for two substrates was independent of each other, and there are two active sites on the single protein that are independent of each other.

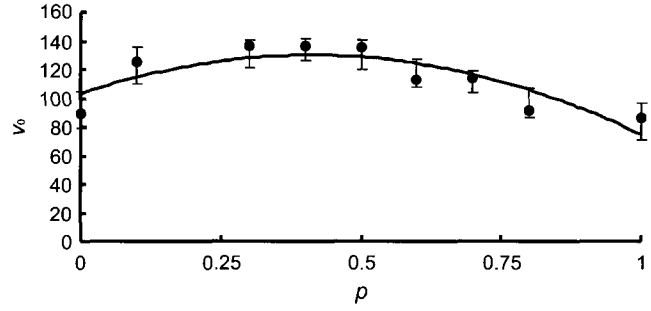

Fig. 1. The competition plot for the reaction of DXAMase with dextran and starch.

The x-axis is the proportion of starch (p) from 0 to 1 and the y-axis is the rate of reaction (v_o). The total rate of reaction was measured with mixtures of substrates at concentrations (1-p) a_o and pb_o, where the reference concentrations a_o (of dextran) and b_o (of starch) were chosen, respectively, so that they give equal rates in experiments with only one substrate present, and this total rate was plotted against p. If the two reactions occur at the same site, the rate does not change with p; if the two reactions occur at two sites with no interaction, the plot gives a curve with a maximum; if each substrate is more effective at inhibiting the other reaction than in reacting with its own substrate, the plot gives a curve with a minimum [3]. The reaction velocities, v_o (μmole maltose equivalent cleaved/min/mg) with varying amounts (p) of dextran and starch, generated a curve with a maximum, which indicates the enzyme has two independent active sites for the reaction with dextran and starch. The reaction velocity (v_o) was an average calculated from triplicate samples from two independent experiments.

Bacterial films, that adhere to the surface of teeth and dental plaque, are composed of closely packed bacteria in a matrix of two polysaccharides, dextran and mutan [6]. The two polysaccharides contribute about 20% of the dry weight of dental plaque. The formation of these sticky polysaccharides, that have a high affinity for the hydroxyapatite found in teeth, is also the major determinant of cariogenicity [6-8, 10, 23]. Structural studies of the *S. mutans* glucans produced *in vitro* have shown that they mainly have α-(1→3)- and α-(1→6)-glycosidic linkages, with some α-(1→4)-glycosidic linkages [6-8, 10, 23]. Thus, the mutanolytic, amylolytic, and dextranolytic activities are required for efficient removal of dental plaque. The DXAMase has all three of these enzymatic activities [24]. In a model system, using *S. mutans*, the DXAMase was able to inhibit or prevent plaque formation, and substantially remove pre-formed plaque. DXAMase also showed elevated activity on insoluble-glucan, compared to *P. funiculosum* dextranase, and was strongly bound to hydroxyapatite. The enzyme is relatively easy to produce in substantial quantities, using a cheap carbon source, starch.

Acknowledgment

This work was supported by a grant of the Korea Health R&D Project (02-PJ1-PG3-21101-0001), Ministry of Health and Welfare, Republic of Korea.

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