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CHARACTERIZATION OF *PSEUDOMONAS* ISOAMYLASE BY ITS ACTIONS ON AMYLOPECTIN AND GLYCOGEN: COMPARISON WITH *AEROBACTER* PULLULANASE

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SUMMARY

The debranching actions of crystalline isoamylase (amylopectin 6-glucanohydrolase, E.C. 3.2.1.9) of *Pseudomonas* SB15 on amylopectins and glycogens were studied by determining the increase in reducing power and coloration with iodine and by fractionating digests on a Sephadex column. These actions were compared with those of crystalline pullulanase of *Aerobacter*. On incubation, the branching linkages in 1 g of waxy maize amylopectin were completely hydrolyzed by 0.01 mg of the isoamylase in 20 h, while they were not completely hydrolyzed even in 24 h by 1.3 mg of the pullulanase, although 13 mg caused complete hydrolysis in 10 h. On incubation with oyster glycogen, complete hydrolysis of the branching linkages was achieved in 24 h by 0.027 mg of the isoamylase, while 29 mg of the pullulanase caused about 30% hydrolysis of the linkages in 24 h.

Analyses of the debranched glucans produced after various times of incubation, suggested that the isoamlyase hydrolyzes both inner and outer branching linkages of amylopectin, while the pullulanase hydrolyzes outer linkages of amylopectin well but scarcely affects inner linkages. A significant difference between the actions of two enzymes on glycogen was observed: the isoamylase hydrolyzed all the branching linkages comparatively well while the pullulanase hydrolyzed relatively few.

INTRODUCTION

Previously we reported the discovery, purification and properties of *Pseudomonas* isoamylase (amylopectin 6-glucanohydrolase, EC 3.2.1.9)[1,2,3]. This enzyme hydrolyzes all 1,6-glucosidic inter-chain linkages in amylopectin and glycogen. Subsequently, we investigated the distribution of the linear α-1,4-linked unit chains in amylopectins and glycogens by debranching them with the enzyme[4,5]. There are many reports of studies[6,7,8,9] on the fine structure of amylopectin and glycogen using *Aerobacter* pullulanase. In this work, the time courses of hydrolysis of α-1,6-glucosidic

linkages in amylopectin and glycogen using various amounts of *Pseudomonas* isoamylase were compared with those using *Aerobacter* pullulanase.

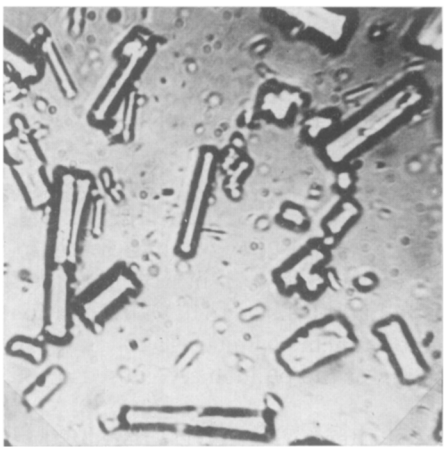
MATERIALS AND METHODS

Enzymes

Crystalline *Pseudomonas* isoamylase was prepared by dropwise addition of $(NH_4)_2SO_4$ solution in the cold to a solution of enzyme[3] purified from the culture filtrate of *Pseudomonas* SB15. Crystalline pullulanase was prepared from the culture filtrate of *Aerobacter aerogenes* ATCC 9621 by the method of Wallenfels and Rached[10].

Substrates

Waxy maize amylopectin, obtained from Nihon Shokuhin Co., was defatted by boiling it with 85% methanol. Oyster glycogen was purchased from Sigma Chemical Co.



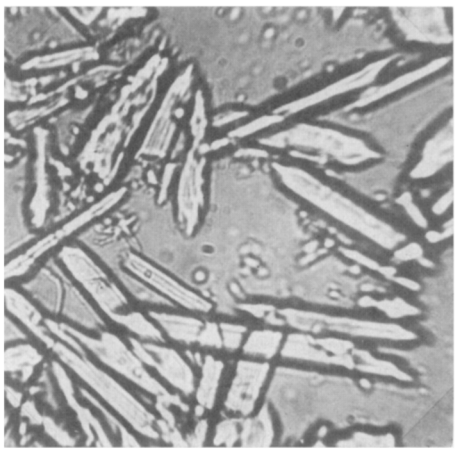


Fig. 1. Crystalline isoamylase from *Pseudomonas* sp., strain SB15.

Fig. 2. Crystalline pullulanase from *Aerobacter aerogenas* ATCC 9621.

Enzyme actions on amylopectin and glycogen

1 g of waxy maize amylopectin or oyster glycogen was incubated with *Pseudomonas* isoamylase in 0.05 M acetate buffer, pH 3.5, or with pullulanase in the same buffer, pH 5.5 and 40 °C, in a total volume of 100 ml. At intervals, aliquots of 7 ml of reaction mixture were taken and mixed with 1 ml of 0.1 M NaOH to inactivate the enzyme.

Estimation of hydrolysis of α-1,6-glucosidic linkages

Reducing power in 1 ml samples of digests was measured by the method of Somogyi–Nelson.

Coloration of the digests with iodine

Volumes of 0.1 ml of digests containing 1 mg of sugars were mixed with 0.5 ml

of 0.4% iodine in 4% aqueous KI. With digests of glycogen, 2.5 ml of 0.4% iodine in 4% KI was used, since the amount of iodine used for amylopectin in the method of Archibald *et al.*[11] was not enough to cause maximum coloration in digests of glycogen. The mixtures were diluted to 10 ml with water and used for determination of the spectra of the iodine complex. The color was measured in a Hitachi 124 UV-VIS spectrophotometer, using a cell of 1 cm light path.

Fractionation of the enzymatic digests on a Sephadex G-75 column

Volumes of 5 ml of digests were neutralized with 0.1 M HCl and submitted to gel filtration on a Sephadex G-75 column (2.5 cm × 90 cm) at 25 °C, as described previously by us[4]. The eluate was collected in 5 ml fractions and the amount of sugar in each was determined by the phenol–sulphuric acid method[12].

RESULTS AND DISCUSSION

The actions of the enzymes on amylopectin and glycogen were examined. The increases of reducing power on incubation of various amounts of crystalline *Pseudomonas* isoamylase and *Aerobacter* pullulanase with 1 g samples of waxy maize amylopectin and oyster glycogen are shown in Figs 3 and 4. Complete debranching of the

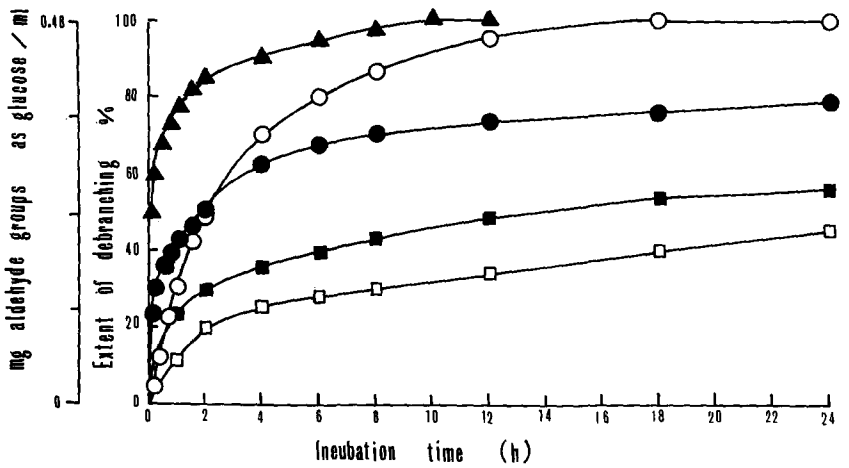


Fig. 3. Debranching actions of the enzymes on waxy maize amylopectin. One gram of waxy maize amylopectin in 0.05 M acetate buffer was treated with 0.001 mg (□); 0.01 mg (○) of isoamylase, pH 3.5, or with 0.13 mg (■); 1.3 mg (●); 13 mg (▲) of pullulanase, pH 5.5, in a total volume of 100 ml. Samples of 1 ml were taken at intervals and their reducing powers were measured as glucose.

amylopectin was achieved in 18 h with 0.01 mg of the isoamylase although a very small amount of α-1,6-linked maltose and glucose stubs may not have been hydrolyzed, as shown previously[3]. In contrast, 1.3 mg of the pullulanase did not cause complete hydrolysis of the branch linkages even in 24 h. However, this amount of pullulanase caused more hydrolysis in 2 h than 0.01 mg of isoamylase. Complete hydrolysis was achieved in 10 h with 13 mg of pullulanase. Thus, some branch

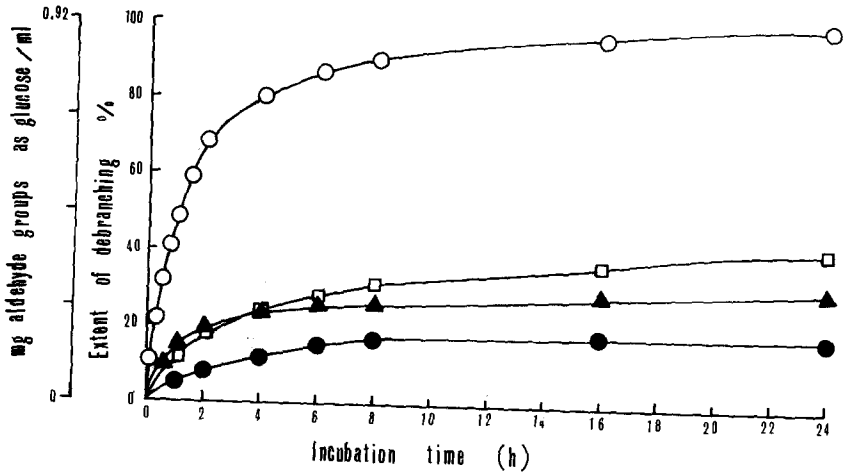


Fig. 4. Debranching actions of the enzymes on oyster glycogen. 1 g of oyster glycogen in 0.05 M acetate buffer was treated with 0.0027 mg (□); 0.027 mg (○) of isoamylase at pH 3.5 or with 2.9 mg (●); 29 mg (▲) of pullulanase, pH 5.5, in a total volume of 100 ml. Reducing powers were measured as shown in Fig. 1.

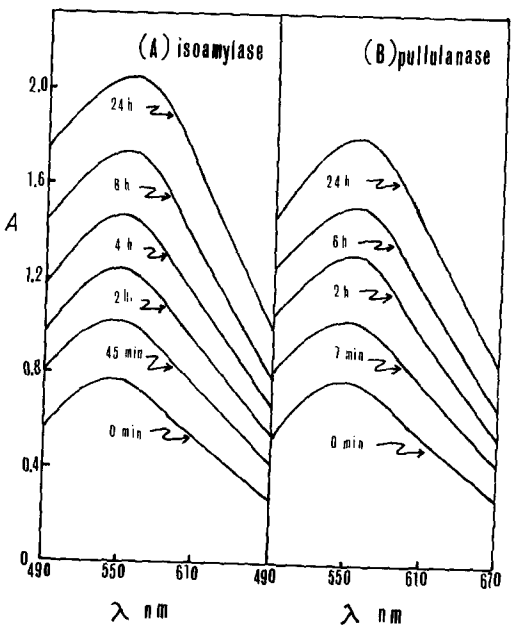


Fig. 5. Increase of iodine coloration of waxy maize amylopectin on treatment with the enzymes. 1 g of waxy maize amylopectin in 0.05 M acetate buffer was treated with (A) 0.01 mg of isoamylase, pH 3.5, or (B) with 1.3 mg of pullulanase, pH 5.5, in a total volume of 100 ml. Samples of 0.1 ml of reaction mixtures were removed at times when about 23%, 50%, 65–70% and 80–85% debranching had been achieved as shown in Fig. 1. The samples were mixed with 0.5 ml of 0.4% iodine in 4% aqueous KI. The mixtures were diluted to 10 ml with water and used for determination of the spectra of the iodine complex.

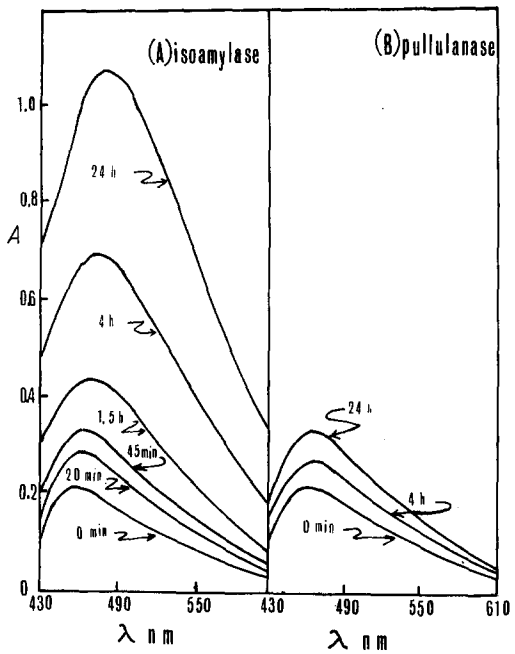


Fig. 6. Increase of iodine coloration of oyster glycogen on treatment with the enzyme. 1 g of oyster glycogen was treated with (A) 0.027 mg of isoamylase, pH 3.5, or (B) with 29 mg of pullulanase, pH 5.5, in a total volume of 100 ml. With both enzymes 0.1 ml samples of reaction mixtures were taken after about 22%, 30–40%, 60%, 80% and 100% of debranching had been achieved, as shown in Fig. 2. Samples were mixed with 2.5 ml of 0.4% iodine in 4% aqueous KI. The mixtures were diluted to 10 ml with water and used for determination of the spectra of the iodine complex.

linkages in amylopectin are not easily hydrolyzed by pullulanase. Glycogen was completely debranched in 24 h by 0.027 mg of isoamylase while about 70% of the branch linkages were not hydrolyzed in 24 h even by 27 mg of pullulanase. About 70% of the linkages in glycogen may be inert to pullulanase. The significant difference between the actions of isoamylase and pullulanase on glycogen was also recognized by β-amylolysis of the enzyme digests[3]. Complete hydrolysis of branching linkages in waxy maize amylopectin and oyster glycogen, respectively, resulted in 48 mg and 92 mg of aldehyde groups as glucose. This seems to be because the average degrees of polymerization of waxy maize amylopectin and oyster glycogen are 23 and 11, respectively[4,5].

The increases in iodine coloration of amylopectin and glycogen due to the action of the enzymes are also shown in Figs 5 and 6. In these experiments 0.01 mg of isoamylase and 1.3 mg of pullulanase were used with waxy maize amylopectin and 0.027 mg of isoamylase and 29 mg of pullulanase with oyster glycogen. Incubation of 0.01 mg of isoamylase with amylopectin for 24 h resulted in about 160% increase in the iodine coloration when complete hydrolysis of the branching linkage was achieved, as shown in Fig. 3. On incubation of 1.3 mg of pullulanase with amylopectin the coloration increased about 120% in the same period. In this case about 80% of the linkages were hydrolyzed, as shown in Fig. 3. Incubation of 0.027 mg of isoamylase with glycogen caused a large increase in the iodine coloration (about 400%)

while incubation with 29 mg of pullulanase caused only about 60% increase in 24 h. In the former case complete hydrolysis of the branching linkage was achieved but in the latter case only about 30% scission occured, as shown in Fig. 4. Previously we reported[3] that hydrolysis of all the branching linkages in oyster glycogen caused about 20% increase in the iodine coloration. However, in previous experiments too little iodine was used to give a maximum iodine value with digests of glycogen. Much more iodine seems to be required to obtain the maximum iodine coloration with debranched products from glycogen than with the products derived from amylopectin. On complete hydrolysis of amylopectin, the maximum absorbance value changed from 540 to 560 nm and on hydrolysis of glycogen from 450 to 480 nm.

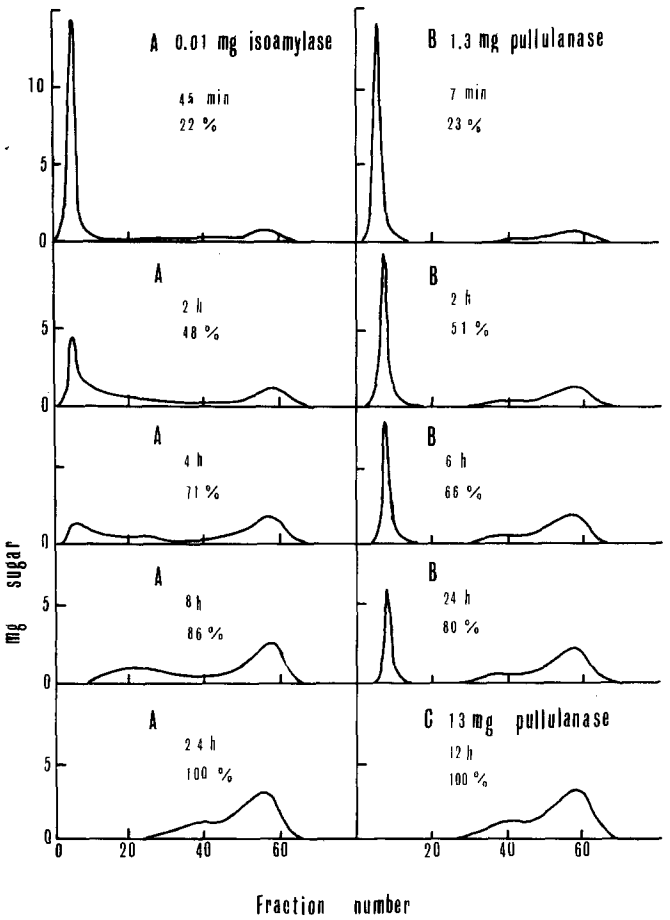


Fig. 7. Fractionation of waxy maize amylopectin on a Sephadex G-75 column after treatment with the enzymes. 1 g of waxy maize amylopectin in 0.05 M acetate buffer was treated with (A) 0.01 mg of isomylase, pH 3.5, (B) with 1.3 mg or (C) 13 mg of pullulanase, pH 5.5, in a total volume of 100 ml. With both enzymes samples of 5 ml of reaction mixture were taken after about 22, 50, 70, 80–85 and 100% debranching had been achieved, as shown in Fig. 1. The samples were fractionated on a Sephadex column as described in the text and the sugars in 5-ml fractions of the eluate from the column were analyzed.

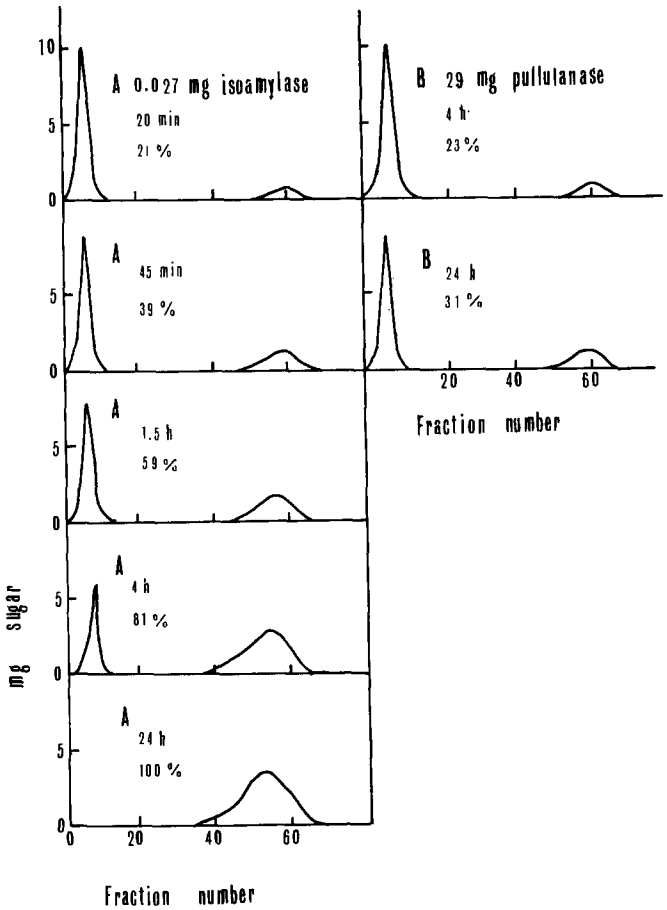


Fig. 8. Fractionation of oyster glycogen on a Sephadex G-75 column after treatment with the enzymes. 1 g of oyster glycogen in 0.05 M acetate buffer was treated with (A) 0.027 mg of isoamylase, pH 3.5, or (B) with 29 mg of pullulanase, pH 5.5, in a total volume of 100 ml. Samples of 5 ml of the reaction mixtures were taken after about 20, 40, 60, 80 and 100% with isoamylase and after 20 and 30% debranching with pullulanase, as shown in Fig. 2. This amount of pullulanase did not give more than about 30% debranching. The samples were fractionated on a Sephadex column and the eluate was collected in 5-ml fractions for sugar analysis.

The A_{max} values of the iodine staining coloration with the original waxy maize amylopectin and oyster glycogen were 0.80 and 0.20, respectively. The A_{max} values of the coloration with digests of the amylopectin and glycogen with isoamylase were 2.0 and 1.1, respectively.

To examine the enzymatic hydrolyses of amylopectin and glycogen in more detail, the hydrolysis products with isoamylase and pullulanase were fractionated using Sephadex G-75, and the sugars in each fraction were measured (Figs. 7 and 8). The digestion products with the two enzymes were withdrawn after similar extents of hydrolysis had been achieved as judged by determination of reducing power, as shown in Figs. 3 and 4. Treatment of amylopectin with 0.01 mg of isoamylase for 45 min or with 1.3 mg of pullulanase for 7 min resulted in about 22% hydrolysis.

With this extent of hydrolysis a small difference was observed between the two fraction patterns of the products with the two enzymes: with isoamylase, fractions contained rather more large glucans having branching linkages and less linear glucans with the residual glucan than those with pullulanase. Isoamylase may act on outer and inner branching linkages. After about 50% or 70% hydrolysis the products with isoamylase clearly contained less residual polymer and more large glucans having branching linkages than the products with pullulanase. After 80–85% hydrolysis with isoamylase the amount of residual polymer had diminished and two peaks appeared, one being linear glucan and the other peak probably linear glucans and glucans containing branching linkages. After 24 h incubation with isoamylase the typical pattern of amylopectin with linear linkages in the products was seen, but after incubation for the same time with pullulanase rather a large amount of residual glucans remained. Incubation with 13 mg of pullulanase for 12 h resulted in complete scission of the branching linkages of amylopectin, giving the same pattern of the linear linkages as that obtained with isoamylase. Successive treatment of waxy maize amylopectin with pullulanase and β-amylase caused 95% (ref. 3) and 92% (ref. 13) conversion of it to maltose. The experiments of Lee *et al.*[14] on the patterns of amylopectin with linear linkages in the products derived with pullulanase indicated that this enzyme does not cause complete hydrolysis of the branching linkages in amylopectin. Complete hydrolysis can be achieved if sufficient enzyme is used, as shown in our present experiments, although a very small amount of α-1,6-linked glucose stubs may remain unhydrolyzed since it is known that these linkages are not cleaved by the enzyme[3,13].

Digests of oyster glycogen obtained with 0.027 mg of isoamylase and 29 mg of pullulanase, respectively, were also fractionated, as shown in Fig. 8. Unlike the case with amylopectin, one clear peak developed after all incubation times with both enzymes. Complete hydrolysis of glycogen by isoamylase gave one peak, as shown previously[4]. Even using about one thousand times more pullulanase than isoamylase, about 70% the residual polymer of glycogen remained.

As reported previously[3], 73% and 68% residual sugars (by weight) remained after treatment of oyster and rabbit liver glycogens, respectively, with pullulanase. In previous work 24 000 units of enzyme were used with 500 mg of glycogen. About 5.8 mg of enzyme protein contains 24 000 units of activity since 29 mg of pullulanase contains about 120 000 units. Drummond *et al.*[15] showed that pullulanase hydrolyzed pullulan in an essentially random manner and could be classed as an endo-enzyme. In our experiments pullulanase looked like an exo-enzyme when acting on amylopectin and glycogen, while isoamylase looked like an endo-enzyme when acting on amylopectin, but like an exo-enzyme when acting on glycogen. However, both isoamylase and pullulanase may act by an endo-mechanism. Pullulanase does not readily penetrate the amylopectin molecule and cannot penetrate the glycogen molecule while isoamylase penetrates amylopectin molecule rather easily but does not readily penetrate the glycogen molecule.

Drummond *et al.*[16] stated that when pullulanase is diluted, its activity towards amylopectin selectively disappears, while its activities towards β- and α-limit dextrins remain. In our experiments no dilution effects were observed, since relatively higher concentrations of the enzymes were used.

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