0273-2289/01/90/0233/$14.25

Cooperativity and Substrate Specificity of an Alkaline Amylase and Neopullulanase Complex of *Micrococcus halobius OR-1*

KAMAKSHI P. RAJDEVI AND GANESA YOGEESWARAN*

Division of Medical Biotechnology, Research and Development, Tamilnad Hospitals Academic Trust-Research Council, Cheran Nagar, Perumbakkam, Chennai 601 302, India, E-mail: gyrdlab@giasmd01.vsnl.net.in

Received January 2, 1999; Revised March 1, 2000; Accepted March 1, 2000

Abstract

The saccharifying alkaline amylase and neopullulanase complex of *Micrococcus halobius OR-1* hydrolyzes both α-(1,4)- and α-(1,6)-glycosidic linkages of different linear and branched polysaccharides. The following observations were made concerning the analysis of the coexpressed amylase and neopullulanase enzymes. Even though the enzymes were subjected to a rigorous purification protocol, the activities could not be separated, because both the enzymes were found to migrate in a single peak. By contrast, two independent bands of amylolytic activity at 70 kDa and pullulanolytic activity at 53 kDa were evident by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), reducing and nonreducing PAGE, and zymographic analysis on different polysaccharides. Preferential chemical modification of the enzyme and concomitant high-performance thin-layer chromatographic analyses of the saccharides liberated showed that amylase is sensitive to 1-(dimethylamino-propyl)-3-ethyl carbodiimide-HCl and cleaved α-(1,4) linkages of starch, amylose, and amylopectin producing predominantly maltotriose. On the other hand, formalin-sensitive neopullulanase acts on both α-(1,4) and α-(1,6) linkages of pullulan and starch with maltotriose and panose as major products. It is understood that neopullulanase exhibits dual activity and acts in synergy with amylase toward the hydrolysis of α-(1,4) linkages, thereby increasing the overall reaction rate; however, such a synergism is not seen in zymograms, in which the

*Author to whom all correspondence and reprint requests should be addressed.
Present address: Division of Biotechnology R&D (III Floor), Sri Ramachandra Medical College and Deemed University, Porur, Chennai 600 116, India.

enzymes are physically separated during electrophoresis. It is presumed that SDS-protein intercalation dissociated the enzyme complex, without altering the individual active site architecture, with only the synergism lost. The optimum temperature and pH of amylase and neopullulanase were 60°C and 8.0, respectively. The enzymes were found stable in high alkaline pH for 24 h. Therefore, the saccharifying alkaline amylase and neopullulanase of *M. halobius OR-1* evolved from tapioca cultivar shows a highly active and unique enzyme complex with several valuable biochemical features.

Index Entries: *Micrococcus*; substrate specificity; amylase; neopullulanase.

Introduction

Starch is an important storage polysaccharide in plants and is a raw material in the food and beverage industries *(1)*. Starch-hydrolyzing enzymes include the endo-α-amylases (EC 3.2.1.1), exo-β-amylases (EC 3.2.1.2), glucoamylase (EC 3.2.1.3), isoamylase (EC 3.2.1.68), pullulanase (EC 3.2.1.41), and cyclodextrin glucanotransferase (EC 2.4.1.19) *(2)*. In recent years, pullulanase has gained importance in starch bioprocessing as a starch-debranching enzyme, and it is generally used in combination with other amylolytic enzymes for the production of various syrups, because it improves the saccharification rate and yield *(3)*. It has already been accepted that pullulanase (Pullulan-6-glucanohydrase, EC 3.2.1.41) can hydrolyze α-(1,6)-glycosidic linkages in pullulan, starch, amylopectin, and related polysaccharides, whereas amylases (1,4-α-D-glucanohydrolase, EC 3.2.1.1) hydrolyze the α-(1,4) linkages specifically *(4)*. True pullulanase does not show activity toward the linear α-(1,4)-linked oligosaccharides, and α-amylase shows no activity against pullulan or branched substrates *(5)*. By contrast, amylopullulanase of *Thermoanaerobacter ethanolicus 39E* (formerly *Clostridium thermohydrosulphuricum* 39 E *[6]*) possesses both α-(1,6) and α-(1,4) activities at a single site *(7)* or at two different active sites as seen in *Bacillus circulans F-2 (8)*. In addition to the aforementioned enzymes of the amylase family, neopullulanase has been reported to be a new type of pullulan-hydrolyzing enzyme from *Bacillus stearothermophilus TRS 40 (9)*. The neopullulanase could efficiently hydrolyze pullulan, but its ability to hydrolyze starch was limited. Further studies revealed that neopullulanase hydrolyzed not only α-(1,4)-glucosidic linkages but also specific α-(1,6)-glucosidic linkages of several branched polymers *(10)*.

During the course of the study on the saccharifying enzyme system of *Micrococcus halobius OR-1*, we purified an amylase and neopullulanase, which behaved differently from other major potent saccharifying amylase and pullulanase reported earlier *(3,11)*. We observed that amylase (A) and neopullulanase (NP) exist as individual molecular forms (A-NP) with α-(1,4) endolytic activity of amylase leading predominantly to maltotriose and α-(1,4) and α-(1,6) activity of neopullulanase producing maltotriose and panose. In this article, we report on the detailed purification, molecular properties, and synergistic action pattern of the two enzymes on different polysaccharide substrates.

Materials and Methods

Materials

Pullulan (MW 65,000), glucose (glc), maltose (glc_2), maltotriose (glc_3), panose-isopanose isomeric mixture (glu_3[I]), and 1-(dimethylamino-propyl)-3-ethyl carbodiimide-HCl (EDAC) were from Sigma (St. Louis, MO). Amylose and amylopectin were from E-Merck (Darmstadt, Germany). All other chemicals were from either Loba Chemie or Hi-Media Chemicals (Mumbai, India).

Bacterial Strains and Growth Conditions

M. halobius OR-1 strains producing saccharifying amylase-pullulanase were used as the source (the enzyme nomenclature of *pullulanase* has been altered to *neopullulanase* in this article based on the mode of action) *(12)*. The bacterium was cultivated in a complex medium containing the following: 2 g/L of $NaNO_2$, 1 g/L of KH_2PO_4, 2 g/L of KCl, 2 g/L of $MgSO_4$, 50 mg/L of $FeSO_4$, 5 g/L of NaCl, 5 g/L of yeast extract, 10 g/L of tryptone, and 20 g/L of polysaccharide. The initial pH of the uninoculated medium was 6.0, and 2% tapioca starch served as the carbohydrate inducer. The culture was vigorously shaken at 120 rpm for 48 h for optimal enzyme production.

Amylase-Neopullulanase Activity

Amylase activity was measured in a reaction mixture containing 0.1 mL of 1% soluble starch in 50 m*M* sodium phosphate buffer at pH 8.0 and 0.1 mL of native or modified enzyme solution. This mixture was incubated at 60°C and samples were aliquoted at appropriate time points. The reaction was stopped by heat treatment for 2 min at 95°C, and the amount of reducing sugar produced was estimated by the Nelson-Somogyi method *(13)* with glucose as the standard. To assay the neopullulanase activity, 1% pullulan was added as substrate instead of starch in the above assay procedure. One unit of enzyme was defined as the amount of enzyme that catalyzed the production of reducing sugar corresponding to 1 µmol of glucose from soluble starch or pullulan used as substrate under defined experimental conditions as outlined for each study.

Protein Determination

Protein concentrations were determined by the Bio-Rad macroprotein assay methods as described by Bradford *(14)* with some modifications. Microassay procedures were performed with crystalline bovine serum albumin (BSA) as the standard protein and assayed using a Microwell Strip-ELISA plate reader (Model EL301), as described by Rylatt and Parish *(15)*.

Preparation of Guar Gum–Starch Affinity Matrix

Guar gum (a polysaccharide of galactose-mannose) was activated with epoxy groups using epichlorohydrin *(16,17)*. For epoxy activation,

10 mL of epichlorohydrin was added to 20 g of guar gum, and to this gel suspension 40 mL of 0.6 *M* NaOH containing 2 mg/mL of potassium borohydride was added. The mixture was finely blended and left at 37°C for 12 h, and the reaction was stopped by washing with deionized water by suction filtration. Then, the matrix was immediately treated with 125 mL of 0.1 *M* NaOH and transferred to a solution of 1.5 g of starch in 60 mL of 0.1 *M* NaOH. To this mixture, 1 mL of epichlorohydrin was added, and coupling of starch to the crosslinked guar gum matrix was allowed to proceed for 16 h at 45°C at 120 rpm. The gel was then suspended in 1 *M* ethanolamine to block the free epoxy groups and left overnight, and the resultant matrix was washed with 0.1 *M* sodium phosphate buffer (pH 8.0) containing 0.5 *M* NaCl, and 0.1 *M* sodium acetate containing 0.5 *M* NaCl with distilled water wash between each step. This gel was then equilibrated with 50 m*M* phosphate buffer, pH 8.0, and used for purification.

Purification of Amylase and Neopullulanase

The *M. halobius OR-1* strain was cultivated for 48 h at 32°C at 120 rpm. A total of 2 L of bacterial culture was centrifuged at 6000*g* for 20 min to pellet down the cells. The supernatant was brought to 60% ammonium sulfate saturation by adding solid ammonium sulfate to the supernatant, and the solution was left at 4°C for 16 h. The resultant precipitate was recovered on centrifugation at 7500*g* for 30 min, redissolved in 50 m*M* sodium phosphate buffer (pH 8.0), and dialyzed at 4°C against the same buffer for 24 h with several changes of buffer. After removal of insoluble matter by centrifuging at 8000*g* for 30 min, the enzyme solution was applied to a DEAE-cellulose column (1.25 × 12 cm; Himedia, India) that was previously equilibrated with 300 mL of 20 m*M* sodium phosphate buffer (pH 7.0). The enzyme was eluted at a flow rate of 0.2 mL/min with a linear gradient from 0.1 to 1.0 *M* NaCl. Fractions (1.0 mL per tube) were collected and their amylase and neopullulanase activity was determined as previously described. The active fractions collected from the DEAE-cellulose column were dialyzed against 20 m*M* sodium phosphate buffer with several changes of buffer and concentrated with a Millisart (Millipore). The concentrated enzyme preparation was applied to a Q-Sepharose column (1.0 × 5 cm) (Sigma) preequilibrated with 20 m*M* sodium phosphate buffer (pH 7.5) and eluted with a linear gradient of 0.1–1.0 *M* NaCl. Fractions of 0.5 mL per tube from the Q-Sepharose column were collected and screened, and then the active fractions were pooled. These fractions were then concentrated and applied to a guar gum–starch-CL affinity column preequilibrated with 50 m*M* sodium phosphate buffer (pH 8.0). The bed volume was 10 mL (2.5 × 2.0 cm). The column was washed with 20 m*M* sodium phosphate buffer and eluted with a gradient of 0.1–1.0 *M* NaCl. The fractions collected (0.5 mL per tube) were dialyzed overnight and used for other analytical experiments.

Enzyme Characterization

The relative molecular mass was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10%) by the method of Laemmli *(18)* using an apparatus from Hoefer (San Francisco, CA). Proteins on the polyacrylamide gel were stained with 0.2% Coomassie brilliant blue R-250. Medium range molecular weight markers (14–97 kDa) were used for the estimation of M_r of the separated proteins.

Activity Staining by Native PAGE

The affinity-purified enzyme was electrophoresed at a concentration of 8 µg/well in the gel. Prior to polymerization of the gels, pullulan, soluble starch, amylose, or amylopectin was added to a final concentration of 1% (w/v). Amylose was made completely soluble by boiling with 5% (v/v) dimethyl sulfoxide in water. After conducting electrophoresis at 4°C, the gels were washed with cold phosphate buffer (pH 8.0) and incubated at 60°C for 20 min for the elucidation of the enzymatic action. For pullulanase activity, staining of the gel was processed as for the determination of glycoproteins in SDS gels (Pharmacia) and developed using Schiff's reagent *(19,20)*. For amylase activity, the reaction was arrested with 0.1 *M* HCl and then stained with a solution of 0.15% iodine: 1.5% KI (w/v) *(21)*.

Preferential Modification of Amino Acids

EDAC Treatment

EDAC was used to modify the aspartate and glutamate residues *(22)*. The reaction was performed by incubating 15 µg/mL of purified enzyme solution with known quantities of EDAC in 200 m*M* ethanolamine (nucleophilic agent) in 50 m*M* sodium phosphate buffer (pH 6.0) at 37°C for 30 min. Enzyme solutions were dialyzed against 50 m*M* sodium phosphate buffer (pH 8.0). The hydrolysis reaction was initiated by adding the modified enzyme to the substrate under standard experimental conditions, and the reaction was terminated by heat inactivation. Residual amylase and neopullulanase activities were determined as described above.

Formaldehyde Treatment

Formaldehyde was used to modify the ε-amino group of lysine by reductive alkylation *(23)*. The purified enzyme (15 µg/mL, pH 8.0) was treated with various concentrations of formaldehyde containing 5 m*M* potassium borohydride incubated at 37°C for 30 min. The dialyzed modified enzyme was used for analyzing the residual amylase and neopullulanase activities.

EDTA Treatment

Varying concentrations of EDTA were added to the purified enzyme (15 µg/mL) and incubated at 45°C for 30 min. The enzyme solution was then dialyzed extensively to remove the EDTA-metal adducts. The result-

ant apoenzyme was added to starch or pullulan under standard reaction conditions, and the remaining amylase and neopullulanase activity was calculated as mentioned above *(23)*.

Chromatographic Analysis of Products

Samples (10 µL) of the reaction mixture were subjected to chromatography on precoated silica-gel high-performance thin-layer chromatographic plates (5 × 5 cm) (Kieselgel; E-Merck) in a solvent system composed of butanol, pyridine, and water (10:4:3 [v/v/v]). Chromatograms were developed by spraying with aniline/diphenylamine reagent, as reported by Ara et al. *(24)*.

Influence of Temperature and pH on A-NP

The relative alkaline amylase and neopullulanase activity was determined at several pH values (4.0–13.0) with 50 m*M* citrate–sodium citrate buffer (pH 4.0–7.0), 50 m*M* sodium phosphate buffer (pH 7.0–9.0), 50 m*M* glycine-NaOH buffer (pH 9.0–11.0), 50 m*M* sodium phosphate–NaOH buffer (pH 11.0–12.0), and 50 m*M* sodium hydroxide–chloride buffer (pH 12.0–13.0). The same buffers were used to determine the pH stability of the enzyme. Ten microliters of enzyme solution was mixed with 10 µL of 10 m*M* buffer, and the mixture was incubated for 1 h at 60°C. After cooling on ice, the sample was diluted 10-fold with 50 m*M* sodium phosphate buffer (pH 8.0) and then used to determine residual enzyme activities. The optimum temperature was determined by assaying the enzyme activity at various reaction temperatures. To determine thermostability, enzyme samples in 50 m*M* sodium phosphate buffer (pH 8.0) were incubated at various temperatures, and then the samples were withdrawn for enzyme assay at appropriate time intervals.

Results

Purification of Amylase and Neopullulanase Complex of M. halobius OR-1

When grown on starch, *M. halobius OR-1* released approx 90% of the enzymes into the culture fluid (data not shown). At the end of 48 h of growth, the crude culture supernatant contained about 1.2 U of amylase activity/mL and 0.92 U of neopullulanase activity/mL *(12)*. After ammonium sulfate precipitation, the enzyme was desalted by dialysis and subjected to further purification by anion-exchange chromatography on DEAE-cellulose. The elution profile showed a single peak of coinciding amylase and neopullulanase activities, and subsequent chromatography on a Q-Sepharose column also resulted in comigrating activities in a single peak. Moreover, the purification by the Q-Sepharose column showed a great increase in the specific activity (Table 1) with purification of 152- and 74-fold of amylase and neopullulanase activity, respectively. The eluate of

Table 1
Purification of Amylase and Neopullulanase[a]

Steps	Total activity (U)		Recovery (%)		Total protein (mg)	Specific activity (U/mg)		Fold		A:NP ratio
	A	NP	A	NP		A	NP	A	NP	
Crude	2400	1840	100	100	2460	0.97	0.7	1.0	1.0	1.3
Ammonium sulfate	2275	1150	94	62	112	20	10	20	14	2.0
DEAE cellulose	1869	678	78	37	20	93	33	96	47	2.8
Q-Sepharose	564	195	65	11	3.8	148	52	152	74	2.8
Guar gum-starch-CL	565	188	65	10	2.2	256	85	264	122	3.0

[a]A total of 2 L of crude culture supernatant was processed for purification. A, amylase activity; NP, neopullulanase activity.

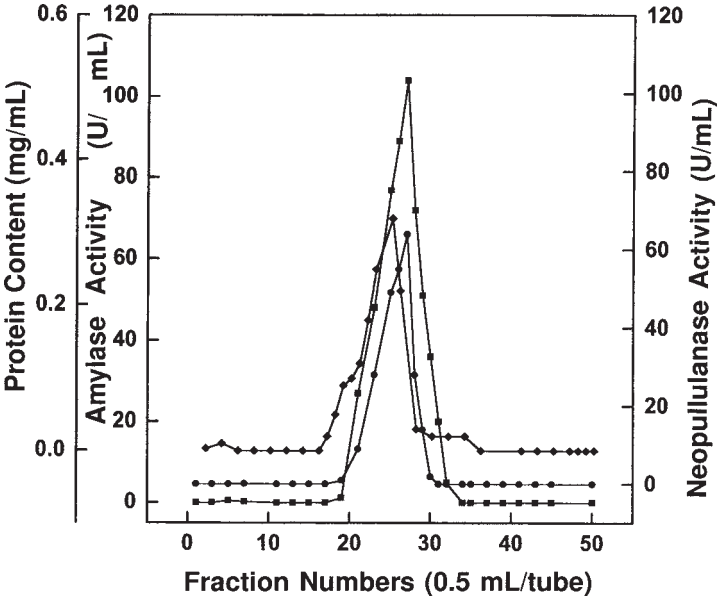

Fig. 1. Elution pattern of A-NP from guar gum-starch-CL affinity chromatography. Detailed experimental conditions are as described in Materials and Methods. (—■—), Amylase activity (U/mL); (—●—), neopullulanase activity (U/mL); (—◆—), protein content (mg/mL).

the Q-Sepharose column showed apparent homogeneity without any interfering bands in SDS-PAGE. Attempts were made to purify and separate the comigrating enzymes through guar gum-starch-CL affinity column chromatography. Figure 1 shows that the two enzymes were not physically separable. Furthermore, a significant increase in the specific activities and A:NP ratios of the copurified amylase and neopullulanase (Table 1) during each step of purification imply that the enzymes are complexed together, although the activity pattern of the two enzymes is different.

Additional experiments were conducted to separate the enzymes by purifying through pullulan or β-cyclodextrin affinity column matrices, but the dissociation of the two activities was not achieved (data not shown). From the results, it could be concluded that the comigrating amylase and neopullulanase enzyme are complexed together through nonspecific intermolecular (ionic, hydrophobic, or hydrogen) bonding and exist as aggregates. However, results from SDS-PAGE showed two individual bands with closely related molecular weights of 70 and 53 kDa, respectively (Fig. 2A). The reducing and nonreducing gel also showed two bands of similar pattern, which signifies that the enzymes were not linked by the disulfide bridges (data not shown). Zymographic patterns shown in Fig. 2B–E on the purified enzymes showed clear activity bands in starch-, amylose-, and amylopectin-incorporated gels at M_r 70 kDa, and an activity band was observed at 53 kDa in the pullulan gel, with no activity seen with

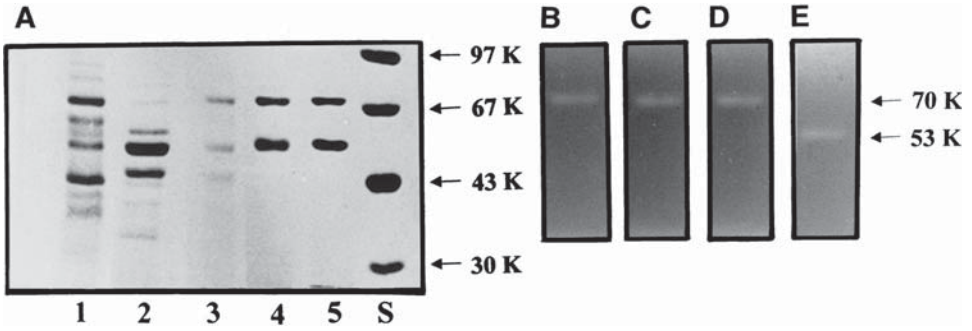

Fig. 2. Electrophoretic profiles of A-NP of *M. halobius OR-1*. Detailed methods for the electrophoretic separation are described in Materials and Methods. **(A)** SDS-PAGE staining for protein by Coomassie brilliant blue: lane 1, crude enzyme preparation; lane 2, ammonium sulfate–precipitated enzyme preparation; lane 3, DEAE-cellulose column eluate; lane 4, Q-Sepharose column eluate; lane 5, guar gum-starch-CL-affinity column eluate; lane S, molecular weight markers, calibration in kilodaltons (97 kDa, phosphorylase B; 67 kDa, BSA; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase). **(B)** Zymogram with starch-incorporated gel; **(C)** zymogram with amylose-incorporated gel; **(D)** zymogram with amylopectin-incorporated gel; **(E)** zymogram with pullulan-incorporated gel. Gel patterns in (B–D) were stained with I_2–KI and (E) was stained with Schiff's reagent as processed for glycoproteins. Migration was from top (cathode) to bottom (anode), and bromophenol blue was used as dye marker. The arrows on the far right indicate the position of the separated amylase (70 kDa) and neopullulanase (53 kDa) protein bands.

other polysaccharides. These results thus suggest that amylase is specifically active on starch, amylose, and amylopectin and that neopullulanase acts on pullulan in the separated forms. It is also inferred that even though neopullulanase was active on pullulan, it was unable to bind with pullulan-affinity matrix, which might presumably be owing to the steric interference incurred by amylase that caused poor/no affinity toward the matrix. By contrast, a differential pattern in the mode of action of the enzymes was seen with the complex proteins in the solution phase.

Mode of Action of Amylase and Neopullulanase

The kinetics of the results given in Fig. 3A show that EDAC is a potent amylase modifier with 73% loss of amylase activity, whereas neopullulanase activity remained unaltered. These observations demonstrate the presence of acidic amino acids such as aspartic and glutamic acids in the active site of amylase. Similarly, neopullulanase lost its activity completely when treated with formaldehyde–potassium borohydride (Fig. 3B), which modifies the ε-amino group of lysine. EDTA treatment (Fig. 3C) tended to inactivate neopullulanase without altering the amylase activity, signifying the involvement of metal ion in the active site of neopullulanase. Hence, amylase and neopullulanase differ widely in their specificity, and it might be inferred that the active sites of amylase and neopullulanase are essentially different.

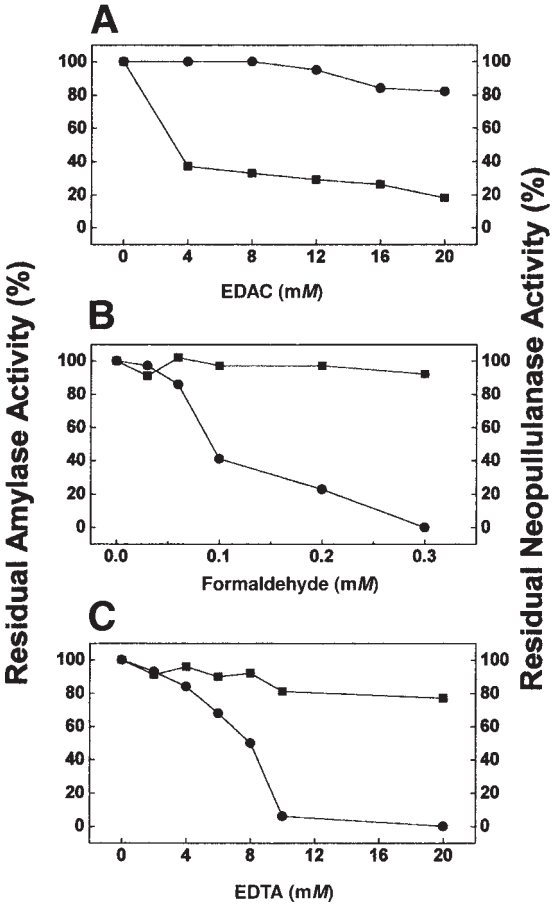

Fig. 3. Effects of **(A)** EDAC, **(B)** formaldehyde, and **(C)** EDTA on amylase and neopullulanase activity. (—■—), Residual amylase activity (%); (—●—), residual neopullulanase activity (%). Detailed procedures are given in Materials and Methods.

In addition, the protective effect of starch and pullulan toward the chemical modification of the A-NP was investigated in detail to study the specificity and mode of action of the *M. halobius OR-1* enzymes. Results from Table 2 imply that neopullulanase was completely inhibited by formaldehyde, in addition to the 24% loss of amylase activity. By contrast, EDAC inactivated 73% of amylase activity without a significant loss in neopullulanase activity. It could thus be justified that neopullulanase acts on both pullulan and starch, whereas amylase acts only on starch. When pullulan was added during EDAC modification of amylase, a 17% residual amylase activity was observed with no significant change in neopullulanase activity. By contrast, on addition of starch, complete protection of the amylase activity site was seen when residual activity was 92%. Similarly, when pullulan was added during formaldehyde treatment, total protection of the neopullulanase site was observed with 87% of residual activity. But the addition of starch resulted in partial protection of the neopullulanase site

Table 2
Protective Effect of Substrates
on Modification of *M. halobius OR-1* Amylase and Neopullulanase

	Residual activity (%)	
Modification	Amylase	Neopullulanase
Unmodified enzyme control	100	100
EDAC treated[a]	27	97
EDAC-modified + pullulan	17	100
EDAC-modified + starch	92	95
Formalin treated[b]	76	0
Formalin treated + pullulan	93	87
Formalin treated + starch	77	58

[a]The purified enzyme (15 µg/mL) was incubated with 5 m*M* EDAC for 30 min in buffer containing 200 m*M* ethanolamine in the presence or absence of 0.3 mg of pullulan or starch per milliliter. The solutions were then dialyzed to remove the EDAC, and the residual activity was assayed as described in Materials and Methods.

[b]The purified enzyme was treated with 0.3 *M* formaldehyde and incubated with 0.3 mg of starch or pullulan per milliliter. The enzyme solution was then dialyzed, and the residual amylase and neopullulanase activity was assayed as described in Materials and Methods.

with 77 and 58% of residual A and NP activities, respectively. In addition, total inhibition of amylase and neopullulanase activities were noted when the EDAC-modified enzyme was further treated with formaldehyde (data not shown). In all of these preferential modification studies, it was found that starch seemed to protect both the amylase and neopullulanase sites, whereas pullulan protected only the neopullulanase site.

These observations were further confirmed by analysis of the reaction products of the enzyme on reaction with starch amylose, amylopectin, and pullulan (Fig. 4). It was inferred that starch, amylopectin, and pullulan predominantly produce maltotriose with traces of panose. However, only maltotriose was obtained on digestion with amylose, an α-(1,4)-linked linear polymer. It has already been reported that the action of the α-amylase of *M. halobius OR-1* is highly endolytic, producing maltotriose as limit dextrins *(12)*. The digestive products of pullulan show the existence of two activities, which could presumably be α-(1-6) hydrolytic activity producing maltotriose and α-(1,4) hydrolytic activity producing panose. Thin-layer chromatography of the formaldehyde-modified enzyme products resulted in the production of maltotriose with starch, amylose, and amylopectin as substrates with no traces of any products with pullulan (Fig. 4). Results also suggest that amylase activity is not affected by formaldehyde reduction. By contrast, the products of the EDAC-modified enzyme show digestive products corresponding to maltotriose when digested with starch and maltotriose and panose with pullulan. Thus, these results strongly imply that the amylase of *M. halobius OR-1* acts on the cleavage of α-(1,4) linkages of starch, amylose, and amylopectin producing maltotriose. Fur-

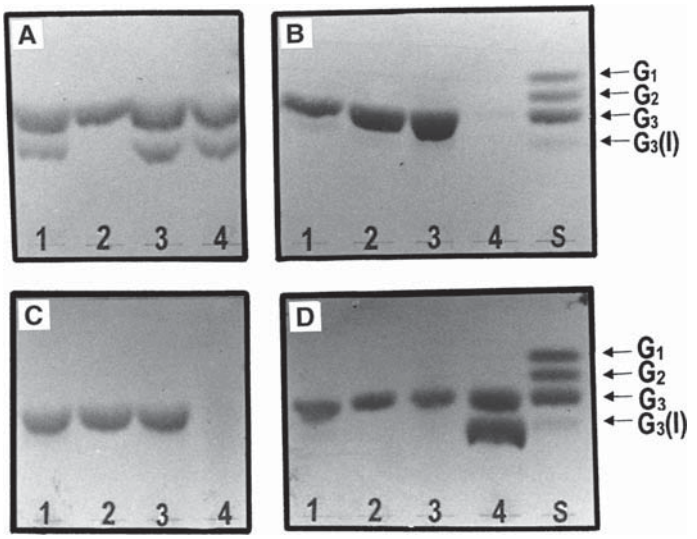

Fig. 4. Thin-layer chromatogram of the sugars liberated by the action of purified *M. halobius OR-1* enzymes. **(A)** Products with the unmodified enzyme; **(B)** products with the formaldehyde-treated enzyme; **(C)** products with the EDTA-treated enzyme; **(D)** products with the EDAC-treated enzyme. Lane 1, digestion with starch; lane 2, digestion with amylose; lane 3, digestion with amylopectin; lane 4, digestion with pullulan; lane S, standard sugars: G_1, glucose; G_2, maltose; G_3, maltotriose; G_3(I), panose. For further details, *see* Materials and Methods.

thermore, the neopullulanase component exhibits dual specificities, located in a single active center, toward the hydrolysis of α-(1,4) and α-(1,6) linkages of starch and pullulan. Thus, it is suggested that the α-(1,4)-hydrolyzing activity of neopullulanase also acts on starch in synergy with the amylase. Such cooperativity increases the overall reaction rate that contributed to the high saccharification rate observed by the *M. halobius OR-1* enzymes. Hence, this enzyme component of *M. halobius OR-1* belongs to the neopullulanase-like enzyme.

Physicochemical Stability of Purified Amylase and Neopullulanase

The results presented in Fig. 5A illustrate that both amylase and neopullulanase activities were active over a broad temperature range from 40 to 80°C, showing optimal activity at 60°C for amylase and 50°C for neopullulanase. The effect of temperature on the heat stability of amylase and neopullulanase in the absence of substrate is shown in Fig. 5B and Fig. 5C, respectively. Both the enzyme activities were entirely stable up to 80°C for 24 h, but a rapid loss in neopullulanase activity was observed beyond 24 h. The half-life of amylase activity was about 36 h at 60°C and 30 h at 80°C. But, in the case of neopullulanase, the half-life ranged from about 30 h to 24 h at 60°C. Interestingly, the stability of the two enzymes ranged between 24 and 36 h at their optimal temperatures.

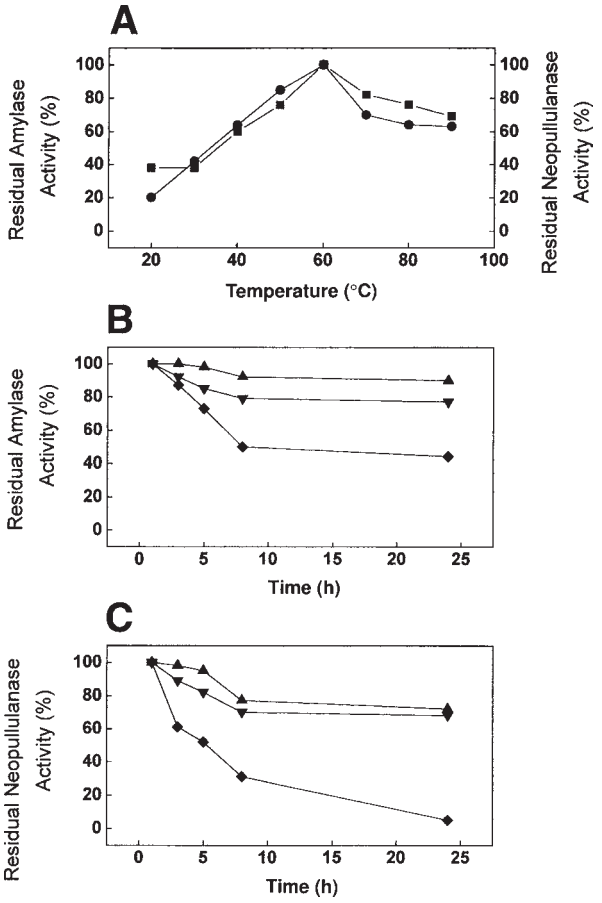

Fig. 5. Optimal temperature and thermal stability of amylase and neopullulanase. **(A)** Optimal enzyme activity ([—■—], residual amylase activity; [—●—], residual neopullulanase activity); **(B)** stability of amylase at different temperatures at different time points; **(C)** stability of neopullulanase ([—▲—], stability at 40°C; [—▼—], stability at 60°C; [—◆—], stability at 80°C). For more details, *see* Materials and Methods.

The pH optima for amylase and neopullulanase (Fig. 6A) shows that the optimal pH of the two enzyme activities were the same (pH 8.0), and both the enzymes showed similar trends of activity over a wide pH range. Both amylase and neopullulanase activities were stable at a wide pH range of 6.0–12.0 (Fig. 6B). Amylase and neopullulanase showed high enzyme activity (above 90%) at an alkaline pH range of 8.0–12.0. However, at pH values below 8.0, the enzyme activities dropped sharply to below 50% of maximal activity. To examine the pH stability, the enzymes were incubated with buffers of various pH values for 1 h at 60°C and the residual enzyme activity was assayed. As shown in Fig. 6B,6C, amylase retained more than 85% of its initial activity at a pH range of 6.0–13.0, whereas neopullulanase retained only 50% of the activity at this range. These pH tolerance results indicated that A-NP may be an extreme alkalophilic enzyme. These obser-

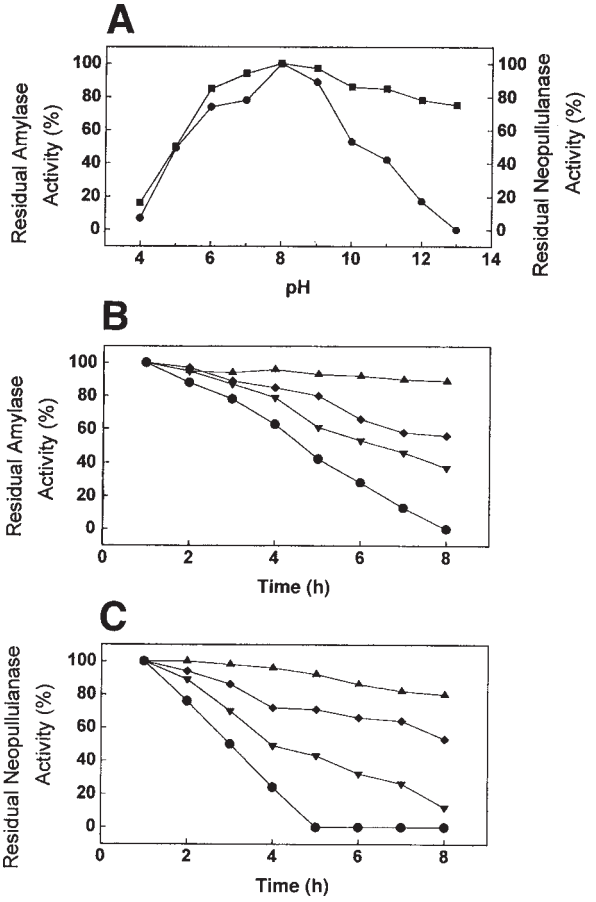

Fig. 6. Effect of pH on enzyme activity and stability on purified *M. halobius OR-1* enzymes. **(A)** Optimal enzyme activity ([—■—], residual amylase activity; [—●—], residual neopullulanase activity); **(B)** stability of amylase; **(C)** stability of neopullulanase ([—▲—], stability at pH 6.0; [—◆—], stability at pH 8.0; [—▼—], stability at pH 10.0; [—●—], stability at pH 12.0). For details of the buffers used, *see* Materials and Methods.

vations further suggest the differential physicochemical characteristic of A-NP of *M. halobius OR-1* enzymes.

Discussion

The coexpressed enzymes from *M. halobius OR-1* were purified and the pure enzyme had both amylase and neopullulanase-like activity with its own unique mode of action. The first description of pullulanase activity was from *Klebsiella pneumoniae (25)*, which specifically hydrolyzed the α-(1,6) linkages of pullulan to yield maltotriose. Other pullulan-degrading enzymes, termed neopullulanases, that specifically cleave α-(1,4) linkages to produce panose and α-(1,6) linkages producing maltotriose have

been reported from *Thermoanerobium* species *(10,26)*. However, the enzymes reported from *Micrococcus Y-1* are capable of hydrolyzing the α-(1-6) linkages in pullulan in addition to cleaving α-(1-4) linkages in starch and other α-(1-4)-linked glucans *(27)*. Nevertheless, these enzymes do not belong to the saccharifying family of amylases. In general, the mechanism of action of *M. halobius OR-1* enzymes is somewhat different from already known saccharifying amylase and pullulanase, from the viewpoint that the high saccharifying rate of *M. halobius OR-1* enzymes was owing to the synergistic action of amylase and pullulanase complex.

During the column chromatography, the elution profiles corresponded almost identically to both amylase and neopullulanase activities, suggesting that both the activities are coinciding in close proximity with each other. Even though we were not successful in separating the two enzyme activities by Q-Sepharose and guar gum/starch crosslinked affinity column chromatographs, the results from SDS-PAGE showed two distinct bands with 70- and 53-kDa molecular weights. It can also be inferred that neither of the two enzymes are subunits nor are they linked by disulfide bonds, as generally seen with proteins of closely related molecular masses. However, they might be linked by other weak ionic interactions, which are disrupted by SDS intercalation. It is also observed from the zymographic pattern that amylase acts on starch, amylose, and amylopectin, whereas neopullulanase acts only on pullulan. The aforementioned findings were further strengthened by studying the dissociation patterns of the enzymes by applying the SDS-treated native enzyme on to the Sephadex G-200 gel filtration column. The eluted fraction showed two well-resolved protein peaks with distinct amylase and neopullulanase activities, signifying that the two enzymes could be dissociated by SDS treatment (data not shown). Furthermore, thin-layer chromatographic analyses showed that the action pattern of amylase follows a random endolytic mechanism with maltotriose as the major end product without the production of glucose or maltose from starch. But, the *M. halobius OR-1* neopullulanase component is apparently active not only on the α-(1,4) and α-(1,6) linkages of pullulan, but also on the α-(1,4) linkages of starch, categorizing this to be a neopullulanase-like enzyme, as reported earlier *(10,26)*. Observations of the chemical modification and studies of the saccharide protective effect imply a synergistic mechanism of action of amylase and neopullulanase toward the hydrolysis of α-(1,4)-glucosidic linkages, and it was understood that such an effect may be responsible for the high saccharification rate as observed earlier *(12)*. It was intriguing to note that such synergistic action could be seen only in the solution phase and not in the separated form of the protein on the polyacrylamide gel. It is therefore justified that such an effect was observed owing to the interference of SDS during electrophoresis, which had separated the two proteins into individual molecular forms with the loss of synergism in their activities. However, it is interesting that the individual activities were not affected at all.

The A-NP of *M. halobius OR-1* had an optimal temperature of 60°C and stability up to 80°C. It was also seen that these enzymes could retain more than 50% of activity for more than 24 h at 80°C. Similarly, the enzymes belong to the group alkalophile in that they could withhold more than 70% of activity at pH 12.0. Such high alkalophilicity in the saccharifying coexpressed amylase and neopullulanase group has not been described in any other organism in the past. It is also clearly demonstrated that amylase involves aspartate and glutamate residues in its active site, whereas neopullulanase involves lysine residues for catalysis. This suggests that the enzymes possess different amino acid architecture, but still the involvement of more than one acidic or basic residue in the catalysis cannot be ruled out. Further studies on the kinetics of chemical modification of the enzymes are needed to locate exactly the amino acids in the active sites. Although, many amylopullulanases have been reported in the past, the saccharifying enzymes of *M. halobius OR-1* reported here is a very distinct enzyme complex with both amylolytic and neopullulanolytic characteristics acting in synergy, which may be a novel finding.

Acknowledgments

This work was supported in part by a grant from Tamilnadu State Council for Science and Technology awarded to Dr. Ganesa Yogeeswaran. We gratefully acknowledge a senior research fellowship from the Council of Scientific and Industrial research awarded to K. P. Rajdevi for doctoral study.

References

1. Juge, N., Svensson, B., and Willianson, G. (1998), *Appl. Microbiol. Biotechnol.* **49,** 385–392.
2. Cornelis, P. (1987), *Microbiol. Sci.* **4,** 342, 343.
3. Norman, B. E. (1982), *Starch-Starke* **10,** 340–346.
4. Plant, A. R., Clemend, R. M., Morgan, H. W., and Daniel, R. M. (1987), *Biochem. J.* **246,** 537–541.
5. Kim, C. H. (1994), *FEMS Microbiol. Lett.* **116,** 327–332.
6. Lee, Y. E., Jain, M. K., Lee, C., Lowe, S. E., and Zeikus, J. G. (1993), *Int. J. Syst. Bacteriol.* **43,** 41–51.
7. Mathubala, S. P., Lowe, S. E., Podkovyrov, S. M., and Zeikus, J. G. (1993), *J. Biol. Chem.* **268,** 16,332–16,344.
8. Sata, H., Umeda, M., Kim, C. H., Taniguchi, H., and Maruyama, Y. (1989), *Biochim. Biophys. Acta* **991,** 388–394.
9. Kuriki, T., Okada, S., and Imanaka, T. (1988), *J. Bacteriol.* **170,** 1554–1559.
10. Imanaka, T. and Kuriki, T. (1989), *J. Bacteriol.* **171,** 369–374.
11. Lee, C., Saha, B. C., and Zeikus, J. G. (1990), *Appl. Environ. Microbiol.* **56,** 2895–2901.
12. RajDevi, K. P. and Yogeeswaran, G. (1999), *World J. Microbiol. Biotechnol.* **15(2),** 223–227.
13. Somogyi, M. (1952), *J. Biol. Chem.* **195,** 19–23.
14. Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
15. Rylatt, D. B. and Parish, C. R. (1982), *Anal. Biochem.* **121,** 213, 214.
16. Gupta, K. C., Sahni, M. K., and Rathore, B. S. (1989), *J. Chromatogr.* **169,** 183.
17. Sundberg, L. and Porath, J. (1974), *J. Chromatogr.* **40,** 87–98.
18. Laemmli, U. K. (1970), *Nature* **227,** 680–685.
19. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochemistry* **10,** 2606–2617.
20. Mathubala, P. S. and Zeikus, J. G. (1993), *Appl. Microbiol. Biotechnol.* 487–493.

21. Carraway, K. L. and Koshland, D. E. (1972), *Methods Enzymol.* **25,** 616–623.
22. Boopathy, R. and Balasubramanian, A. S. (1985), *Eur. J. Biochem.* **151,** 351–360.
23. Itkor, P., Tsukagoshi, N., and Udaka, S. (1989), *J. Ferment. Bioeng.* **68,** 247–251.
24. Ara, K., Igarashi, K., Saeki, K., and Ito, S. (1995), *Biosci. Biotechnol. Biochem.* **59,** 662–666.
25. Bender, H. and Wallenfels, K. (1966), *Methods Enzymol.* **8,** 555–562.
26. Takata, H., Kuriki, T., Okada, S., Takesada, Y., Iizuka, M., Minamiura, N., and Imanaka, T. (1992), *J. Biol. Chem.* **267,** 18,447–18,452.
27. Kim, C. H., Choi, H. I., and Lee, D. S. (1993) *J. Ind. Microbiol.* **12,** 48–57.