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Lactobacillus plantarum Amylase Acting on Crude Starch Granules

Native Isoforms and Activity Changes After Limited Proteolysis

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Abstract

The microheterogeneous native amylolytic complex secreted by the isolate A6 of *Lactobacillus plantarum* revealed a selective enzyme specificity loss when submitted to a limited proteolysis under a suboptimum pH condition. A clear electrophoretic profile change toward just one shorter, more acidic, and equally active polypeptide fragment resulted from the pronase E pretreatment. Although the whole enzyme activity remained apparently unaffected for soluble starch, the native parallel activity on intact and non-gelatinized starch granules either from cereals or tubers was dramatically reduced. This phenomenon was more clearly documented by scanning electron microscopy using the easiest accessible native substrate: wheat starch granules. The anion-exchange-purified native enzymes from *L. plantarum* displayed a different optimum pH curve when compared with the thermotolerant α-amylase from *Bacillus licheniformis*. The α-amylases from the lactic-acid-producing A6 isolate presented an electrophoretic profile easily distinguishable from those from *B. liqueniformis* and *B. subtilis* species.

Index Entries: *Lactobacillus plantarum*; amylase; crude starch depolymerization; proteolysis; amylase zymogram.

Introduction

Starch follows cellulose as the second most plentiful source of polymeric glucose in nature. There are several biotechnological approaches for

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the release of the monomeric unit from starch either as such or as its dimer, maltose. The innermost and compact architectural assembly of both starch polyglucose fractions, amylose (linear) and amylopectin (ramified), results in the water-insoluble starch grains as seen in the several cereal and tuberous starch sources. Fungal and bacterial amylases act quite efficiently on gelatinized starches, producing either maltose (α-, β-amylases) or free glucose (amyloglucosidases or glucoamylases). Less often, microbes can produce amylolytic activity acting on native or nonpregelatinized starch granules, e.g., some *Bacillus (1)* and *Streptomyces (2)* amylases. Enzymes degrading solid substrates have a typical bifunctional molecular structure with a catalytic domain linked to a substrate-binding domain through a connection arm, as verified for cellulase and chitinase *(3)*, xylanase *(4)*, and in the particular case of *Aspergillus* glucoamylase *(5)*. However, having a starch-binding domain is apparently not always necessary for an amylase to act on raw starch, e.g., α-amylase from *Bacillus licheniformis (6)*. The search, characterization, and potential industrial uses of this class of glucogenic or maltogenic amylases acting on raw starch granules is an interesting prospect because a significant savings in energy is obtained when starch pregelatinization no longer applies.

A *Lactobacillus plantarum* isolate (A6) producing lactic acid from polymeric starch was described a few years ago *(7)*. The genome fragment involved in the enzyme expression was more recently explored and shown to display an unusual 3' end structure. Since a relatively high homology was deduced when comparing a gene segment (e.g., NH_2 terminus) to that of *Lactobacillus amylovorus* amylase, a common ancestor was proposed as valid for both lactobacilli although qualitative and quantitative differences were seen at the tandem repeats (COOH terminus) *(8)*.

Another unusual feature is now being reported since we realized that in vitro limited proteolysis may dramatically change the *L. plantarum* amylase action mechanism, which may also reflect what may happen in the in vivo process.

Materials and Methods

Bacterial Strain and Culture Conditions

The *L. plantarum* isolate, previously named A6, was obtained from rotting cassava roots *(9)*; it is routinely maintained in glycerol at –80°C as stock culture. Periodic culture check was carried out through the Gram-positive stain and halo-forming ability on agar-solidified cassava starch flooded with diluted iodine/iodide after 24 h of culture at 30°C.

Bacterial growth and amylase production was carried out using a 2-L bioreactor (LSL-Biolafitte, Saint Germain en Laye, France) at 30°C and agitation at 300 rpm. A modification of MRS medium *(10)* with 40 g/L of soluble starch replacing glucose was adopted, and the fermentation pH was maintained at 6.0 by automatic addition of a 5 *N* NaOH solution.

Exocellular Enzyme Recovery and Purification

The cell-free medium from 24 h of growth was cooled and enough solid ammonium sulfate was added under gentle agitation to crop the enzyme precipitated between 50 and 70% salt saturation. The protein precipitate was recovered by centrifugation (15,000*g* for 30 min at 4°C), dissolved in 50 m*M* (pH 6.8) sodium phosphate buffer, and concentrated through a PM-10 (Amicon, W. R. Grace and Co., Danvers, MA) ultrafiltration membrane to one tenth of the initial volume. The retentate was applied to a DEAE-cellulose column (total volume, V_t, 100 mL), and then eluted with a progressive gradient of NaCl up to 1 *M* in the same buffer. The active fractions were pooled, concentrated in the same ultrafiltration assembly, and kept at –30°C.

Analytical Methods

Protein was measured by the Bradford *(11)* method using bovine serum albumin as standard. Amylase activity was assayed incubating 0.1 mL of appropriately diluted enzyme solution with 0.8 mL of a solution containing 1.25 g% of Prolabo soluble starch in 100 m*M* (pH 5.5) citrate-phosphate buffer at 55°C. The reaction was stopped by the addition of 0.1 mL of 2 *N* H_2SO_4 (for iodine/iodide chromogenic reaction) or 2 *N* NaOH (for reducing power estimation). The residual starch was spectrophotometrically quantified at 620 nm after staining with the iodine reagent *(12)*. Released reducing sugars were quantified with the dinitrosalicylate reagent *(13)*. An amylase enzyme unit was defined as the amount of protein allowing the complete hydrolysis of 10 mg of soluble starch in 30 min. Total sugars were measured using the phenol-sulfuric acid method *(14)*. Lactic acid, the main catabolite from starch depolymerization and fermentation, was estimated by NaOH titration and further confirmed by high-performance liquid chromatography on a Shimadzu C-18 column (Shimadzu Co., Tokyo, Japan) with 25% aqueous acetonitrile as mobile phase and a Differential Refraction Index at the recorder unit. Polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Laemmli *(15)* in duplicated 10% gels using a 100 m*M* (pH 8.0) Tris-glycine running buffer. After electrophoresis, the gel was incubated for 1 h at room temperature in the 1% soluble starch containing 100 m*M* (pH 5.5) citrate-phosphate buffer. After a rinse with distilled water, gel flooding with diluted iodine/iodide (0.026/0.26g%) was used to reveal the discoloration zones corresponding to amylase bands. For a comparative purposes, *B. licheniformis* and *B. subtilis* amylases from Sigma (St. Louis, MO) were analyzed under the same conditions.

In Vitro Proteolysis

L. plantarum A6 α-amylase was submitted to in vitro proteolysis with an alkaline protease (Pronase E from *Streptomyces griseus*; Sigma). Five microliters of protease (1 mg/mL) under a suboptimum pH condition for

proteolysis (100 m*M*, pH 5.5, citrate-phosphate) was added to 250 μL of amylase solution (0.5 mg of total protein). Following proteolysis for 12 or 24 h at 25°C, the amylase digest was frozen (–30°C) until used in the electrophoretic procedure. A parallel amylase incubation without protease addition was also performed to obtain the control, also frozen.

Hydrolysis and Scanning Electron Microscopy of Crude Starch Granules

Crude starch granules from different sources (corn, wheat, potato, and cassava) were separately submitted to untreated and preproteolyzed α-amylase action. To 10 mg/mL of each buffered crude starch the conveniently diluted enzyme was added. The incubation temperature was 35°C, and aliquots were collected at predetermined reaction times. After centrifugation, each supernatant was analyzed for total sugars and the precipitate was examined by optical microscopy, which guided the sample selection for the next step. Following glutaraldehyde fixation, a progressive dehydration was carried out with 50, 70, 90, and 100% ethanol. The control and enzyme-treated organosolvent-free starch granules were submitted to homogeneous gold metallization. Scanning electron microscopy (SEM) micrographs were obtained using a Philips XL 30 (Eindhoven, The Netherlands) scanning electron microscope operating at an accelerating voltage of 10 kV at the Electronic Microscopy Center from UFPR.

Results and Discussion

Growth and Enzyme Production of L. plantarum *A6*

The bacterial growth and amylase secretion proceeded quickly and attained their maxima in 1 d, as shown in Fig. 1. The last doubling of bacterial biomass occurred between 14 and 24 h and enzyme titer doubled faster in the last 4 h, respectively, considering that the stationary phase of growth was reached at 24 h of growth. Accordingly, the maximum production of lactic acid and the carbon source depletion here both also achieved at about 24 h of growth.

Amylase Purification

The culture medium supernatants from five fermentation runs (24 h at 30°C) were individually monitored for amylase secretion, pooled, precipitated, desalted, and concentrated by ultrafiltration. Figure 2 shows the protein and enzyme fractionation profiles by anion exchange of the ultrafiltrated retentate. The protein population was resolved in three main blocks: basic or neutral in the column void volume, acid (salt gradient between 0.2 and 0.45 *M*), and more acid (salt gradient about 0.6 *M*). The first and third protein blocks proved devoid of enzymatic action. All amylase activity was sharply obtained in the last protein peak from the midacidity protein block (column fractions of 530–600 mL; NaCl ion strength at 0.45 *M*), a superior result when compared to a previous purification attempted

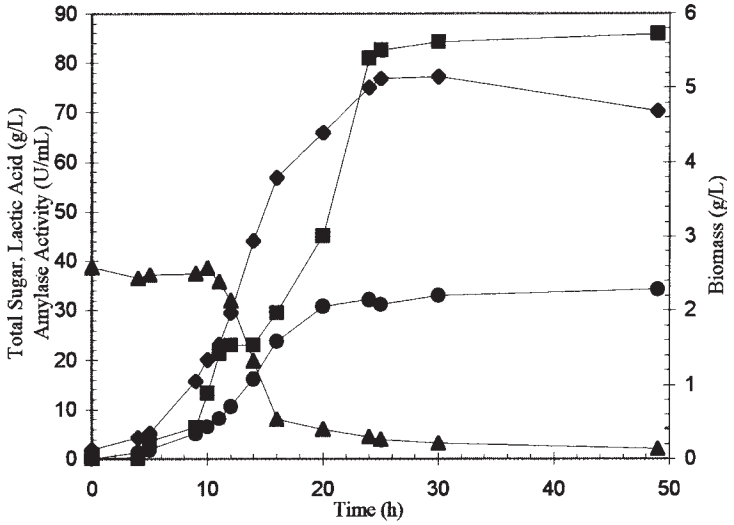

Fig. 1. *L. plantarum* A6 growth and fermentation of soluble starch. ●, lactic acid; ▲, total sugars; ■, amylase activity; ◆, biomass.

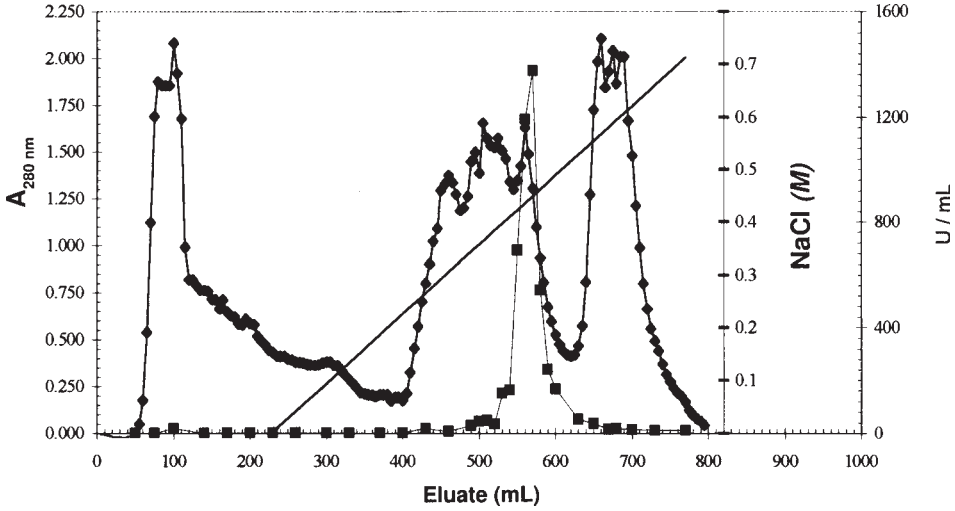

Fig. 2. Chromatographic profile of *L. plantarum* amylase on DEAE-cellulose column. Elution with 50 m*M* (pH 6.8) sodium phosphate buffer and an NaCl gradient (/), at 2.5 mL/min at 5°C. ◆, protein; ■, amylase activity.

under the same chromatographic procedure *(16)*. The active amylase pool was further concentrated by ultrafiltration.

The enzyme fractionation and purification achieved from the cell-free culture medium supernatant until the anion-exchange chromatography step can be summarized as follows: 3.2% total protein recovery (2626 mg to 84 mg), 58.6% yield (677,286 U to 397,085), and 18.5 times as purification factor (specific activity of 259 U to 4794 U/mg of protein).

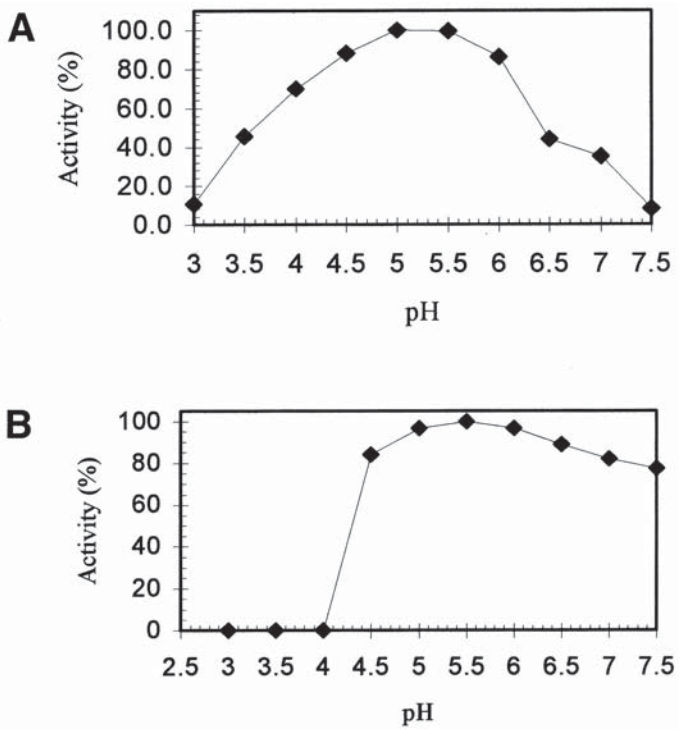

Fig. 3. **(A)** pH effect on *L. plantarum* amylase activity; **(B)** pH effect on *B. licheniformis* amylase activity.

pH Effect on Amylase Activity

Comparative curves for *L. plantarum* A6 and *B. licheniformis* amylases over a broad pH range were obtained (Fig. 3A,B). The second enzyme displayed full activity from pH 4.5 to 7.5 (optimum at pH 5.5), but no activity could be observed below pH 4.0. Conversely, *Lactobacillus* amylase revealed a much broader pH range (optimum at pH 5.0) since more than 40% of the activity could still be detected at pH 3.5, probably reflecting a physiological adaptation of *L. plantarum* to the acid environment generated by the coproduction of large amounts of lactic acid.

L. Plantarum α-*Amylase Activity on Native and Soluble Starches*

Native and preproteolyzed amylase activities were quantified in duplicate assays against soluble starch. Because enzyme activity reduction for soluble starch does not exceed 5%, the limited proteolysis ensured by the suboptimum pH condition in fact did not affect the amylase ability concerning depolymerization capability on soluble starch. On the other hand, when crude and intact starch granules were the alternative substrate, regardless of the botanical source (wheat or corn for cereals, potato or cassava for tubers), almost no amylolytic activity was found, as indicated by the measurements carried out either with the iodine/iodide chromogenic

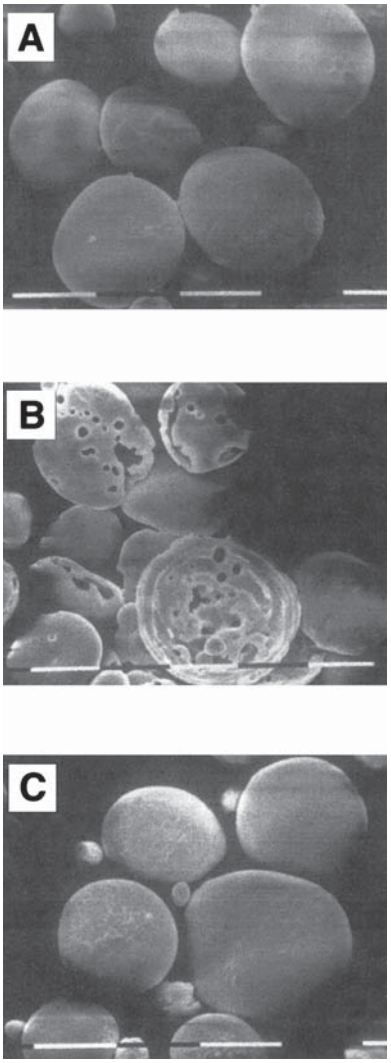

Fig. 4. SEM micrographs of wheat starch granules. **(A)** Untreated granules (bar = 10 μm); **(B)** native amylase-treated granules; **(C)** proteolyzed amylase-treated granules.

reagent or with the reducing power assay. Hence, the net effect of proteolysis is to abolish the recognition and/or binding of the enzyme to the multilayer assembly of amylose and amylopectin in the intact starch granules. SEM was elected in order to see, at a macromolecular level, the enzyme(s) action performance, namely, how much the crude starch granule architecture was in fact damaged. This comparison refers to homogeneous catalysis (native soluble enzyme, soluble starch, full depolymerization measured as generated reducing power or loss of iodochromogeny) in opposition to a suggested defective or null amylolysis in the case of the heterogeneous catalysis (native or proteolyzed soluble enzyme, insoluble starch granules, SEM observation). The images (e.g., Fig. 4A–C; wheat starch granules as

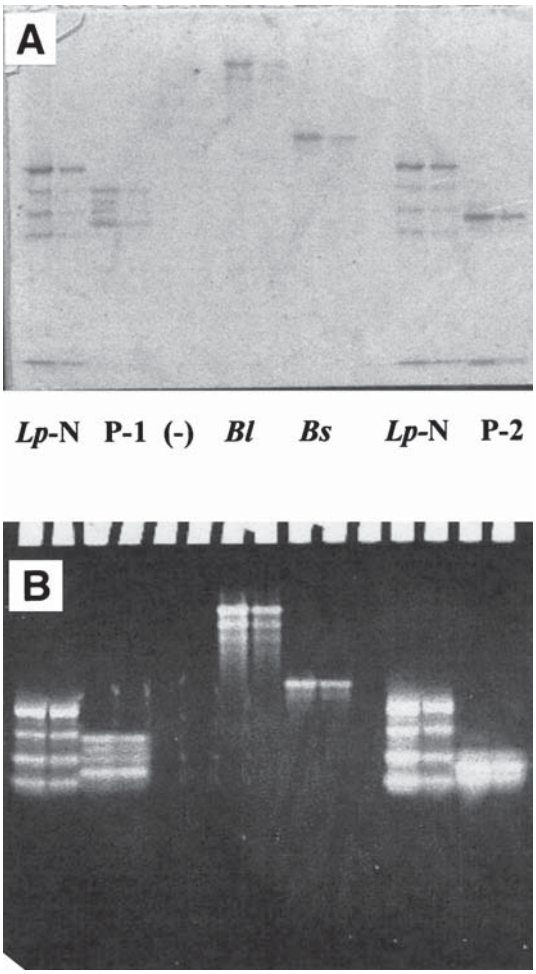

Fig. 5. PAGE zymogram for amylases. **(A)** Gel stained with Coomassie; **(B)** gel replica flooded with dilute iodine/iodide after incorporation of low molecular weight soluble starch and incubation under optimum conditions for amylolysis. Lane *Lp*-N, native *L. plantarum* amylase complex; lane P-1, *Lp*-N after short proteolysis; lane (–), protease control; lane *Bl*, *B. licheniformis* amylase; lane *Bs*, *B. subtilis* amylase; lane P-2, *Lp*-N after longer proteolysis.

model) show pits and corrosion on the surface of wheat starch granules treated with native amylase. In the case of more deeply attacked granules, the multilayer assembly of amylose and amylopectin is still seen in the remaining outermost portion of the granule (Fig. 4B). On the other hand, starch granules incubated at the same conditions (30°C for 24 h or more) with the proteolyzed amylase seems more like the control (incubated granules with no enzyme).

A preliminary inspection by thin-layer chromatography on the nature of the released free sugars indicated maltose as the main product when

the native *L. plantarum* amylase(s) was incubated with either crude or soluble starches.

Amylase Electrophoretic/Zymogram Patterns

PAGE runs (soluble, low molecular weight starch incorporated in the gel duplicate for zymogram) revealed the occurrence of four major bands both for Coomassie staining and for amylase activity. The slow-migrating band (higher molecular weight and/or less acidic p*I*) displayed the highest activity (Figs. 5A,B; lane *Lp*-N). Under matched in vitro preproteolysis, just differing in the incubation time kinetic parameter, the realized tendency is to generate a dominant new enzyme isoform. Its migration behavior was compatible with a reduced molecular weight (Fig. 5A,B; lanes P-1 and P-2) (also verified with a denaturating SDS-PAGE gel; result not shown). An alternative or cumulative explanation could be a more acidic character than at least that of the three of the four original precursors since its banding pattern (Fig. 5A,B; lanes P-1 and P-2) is exactly in the half migration way of the two faster original bands (Fig. 5A,B; lanes *Lp*-N). As an overall view, *L. plantarum* amylolytic complex is completely different from the similar enzymes from either *B. licheniformis* or *B. subtilis* (Fig. 5A,B; lanes *Bl* and *Bs*).

Since the nondenaturating electrophoresis of different native enzyme batches revealed some degree of variation between band intensities (also comparable, with some differences, with data in ref. *16*), a possible proteolysis-provoked physiological mechanism of band interconversion may be operating in the *L. plantarum* amylolytic apparatus.

This hypothesis is being examined through the preincubation of the native amylase family under more accurate kinetic monitoring. Also under examination is the particular macromolecular aspect of intact starch granules from several sources following treatment with native or time/pH-dependent proteolyzed amylase complexes from *L. plantarum*.

Conclusion

A mild acid-tolerant behavior for the α-amylases isolated from *L. plantarum* A6 was described as well as the natural occurrence of isoenzymic forms for such enzyme activity. Proteolysis of these amylases selectively affected their capability of recognition of, binding to, and/or depolymerization of intact starch granules whereas the amylolysis of soluble starch remained almost unaffected.

Acknowledgments

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