Synergistic Hydrolysis of Crude Corn Starch by α-Amylases and Glucoamylases of Various Origins

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ABSTRACT

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Four α-amylases and two glucoamylases from various sources, in eight combinations, were used to study the synergistic hydrolysis of crude corn starch at various temperatures. At 40 and 50°C, the combinations containing *Rhizopus* mold glucoamylase enhanced hydrolysis of corn starch compared wth that obtained with the combinations from *Aspergillus niger*. At 60°C, *Rhizopus* mold combinations gave low reaction yields as the enzyme was inactivated. The differences observed between α-amylases are smaller, with the exception of *Bacillus licheniformis* α-amylase, which presented more than twice the productivity of the other α-amylases, at all temperatures. In terms of substrate conversion at 5 hr of hydrolysis, the combination of *B. licheniformis* α-amylase with *Rhizopus* mold glucoamylase at 50°C presents 76% substrate conversion, whereas, with all the other combinations, starch conversion was 13–73%. HPLC analysis of the reaction products obtained at 50°C showed that the main product of corn starch hydrolysis was glucose at 85–100%. Further experiments showed that *A. niger* glucoamylase and *B. licheniformis* α-amylase were the only enzymes that retained their initial activity after incubation at the temperatures studied.

Starch is an important raw material for the food industry. The production of glucose syrups from starch hydrolysis is a two-stage procedure. Liquefaction takes place in the first stage, where starch is gelatinized and treated by thermostable α-amylase (usually from *Bacillus licheniformis, B. amyloliquefaciens,* or *B. stearothermophilus*) at 90–110°C, pH 6–7. Saccharification takes place in the second stage, where the liquefied hydrolyzate is treated by glucoamylase at 60°C and pH 4–5, until conversion to glucose is stopped at the desired percentage. Even though *Rhizopus* mold glucoamylase is also used, glucoamylase from *Aspergillus niger* is preferred due to better tolerance to the temperatures applied (Kennedy and White 1987; Pancoast and Junk 1990; Nicolov and Reilly 1991). This process has some disadvantages. The reaction occurs in two separate reaction vessels, thus requiring more equipment. The different optimum conditions of the enzymes require conditioning before each step (pH and temperature adjustment), and the time needed to obtain the final product is quite long (about three days). Gelatinization is an energy-consuming stage. And, finally, the high glucose concentration leads to the formation of byproducts (Larsson and Mattiasson 1984; Larsson et al 1989; Pancoast and Junk 1990).

Alternative solutions to this system include enzyme immobilization (Chakrabarti and Storey 1990), membrane reactors (Tachauer et al 1974; Cheryan and Mehaia 1986; Gaouar et al 1997) and aqueous biphasic systems (Larsson and Mattiasson 1984; Larsson et al 1989; Hayashida et al 1990; Liakopoulou-Kyriakides et al 1996; Karakatsanis et al 1997; Karakatsanis and Liakopoulou-Kyriakides 1998). A key feature in these applications is the simultaneous action of α-amylase and glucoamylase on the substrate, known as synergism. According to the studies of Fujii and Kawamura (1985) and Fujii et al (1988) on the synergistic hydrolysis pattern of α-amylase and glucoamylase, α-amylase hydrolyzes glycosidic bonds at the exterior of starch granules, producing nonreducing sugars that remain on the surface of the granule, inhibiting α-amylase from proceeding to the interior of the granule. These sugars are further hydrolyzed by glucoamylase in such a way that it peels away the granule surface, exposing new substrate to α-amylase. The synergistic action results in enhanced reaction yields and increased activity toward crude starch granules, making gelatinization unnecessary. Franco et al (1989) further described the use of a synergistic scheme of the two enzymes for the determination of enzymic activity. They also reported increased glucose and maltose levels in the products, and dependence of the ratio of specific products on the ratio of the enzymes used. Arasaratham and Balasubramaniam (1993) reported increased efficiency of the synergistic hydrolysis of dry-milled corn starch compared to wet-milled corn starch, and Shevel' kova and Sinitsyn (1993) reported dependence of the synergistic action on substrate characteristics and concentration, enzyme concentration, hydrolysis duration, and the mode of α-amylase action.

A large variety of α-amylases and glucoamylases from different sources are available. Therefore, we have investigated whether enzymes from various sources affect glucose production during the synergistic corn starch hydrolysis. Four α-amylases and two glucoamylases in eight combinations were used and the results are presented here.

MATERIALS AND METHODS

Materials

α-Amylases porcine pancreas (PPA) (activity 28 units/mg of solid material), *B. licheniformis* (activity 500 units/mg of protein), *A. oryzae* (activity 40 units/mg of solid material), and *Bacillus* spp. (activity 300 units/mg of solid material) were from Sigma-Aldrich (St. Louis, MO). One unit of activity is defined as the amount of α-amylase that liberates 1 mg of maltose from starch in 3 min at pH 6.9 at 20°C.

Glucoamylases *A. niger* (activity, 6,100 units/mL of liquid) and *Rhizopus* mold (activity, 22,500 units/g of solid material) were also from Sigma-Aldrich. One unit is defined as the amount of glucoamylase that liberates 1 mg of glucose from starch in 3 min at pH 4.5 and at 55°C.

Corn starch was purchased from the local market (Pistofidis, Kilkis, Greece). Dry-milled product of a locally grown dentomorphous corn cultivar contained 11% moisture, 1.45% fat, 6.37% protein, 0,9% ash, and 80.25% total sugars.

Reactions

All reactions described here were conducted in Erlenmeyer flasks employing reciprocal shaking at 150 rpm. The reaction volume was 20 mL. Starch concentration was 5% (w/v) in all experiments, and the enzyme activity was 112 U of α-amylase, 112 U of glucoamylase, or both. The reaction was kept at pH 5.0 with acetate buffer (0.05*N*) and experiments were conducted at 40, 50, and 60°C respectively. The enzyme combinations are given in Table I.

Sample Preparation and Analysis

At given time intervals, a sample (1 mL) was taken, centrifuged at 2,500 × *g* for 3 min (Selecta, Centronic, Spain), filtered through

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0.22-µm filters (Millipore GSWP, Ireland), and kept in a refrigerator before analysis. The total carbohydrate content was determined by the spectrophotometric method of phenol-sulfuric acid (Dubois et al 1956). Starch conversion was calculated from the total sugars measured. Sugars were also determined by HPLC with a Dionex, series 4500i HPLC system, using pulsed electrochemical detection, over a gold electrode constructed in our department and a Hamilton HC-75 column. Water was used as eluent (flow rate 1 mL/min) and 0.3*N* NaOH was added (postcolumn addition), to give a final solution of 0.1*N* NaOH.

Residual Enzyme Activity

Enzymes were incubated at 40, 50, and 60°C at 150 rpm. After 2 hr of incubation, temperature was set to 40°C and 5% corn starch was added. Hydrolysis was conducted for 2 hr and residual activity was determined as the ratio of glucose production to the glucose production by the same enzyme, without prior incubation, at the same temperature.

Michaelis-Menten Kinetics

The K_M and V_{max} values were determined by measuring the initial rates of the reaction of each enzyme with corn starch (2.5–50% w/w). The parameters were calculated by the use of computer software (Enzfitter, Elsevier-Biosoft).

Statistical Analysis

Experiments were performed in triplicate. Values displayed in figures are average values. For the determination of the reproducibility of the results, the coefficient of variation (CV) was used. The differences obtained by these data were further analyzed for statistical significance by the Student *t*-test (Christian and Christian 1994) at the 5% level of probability.

RESULTS AND DISCUSSION

All the enzymic combinations included in Table I were used for the synergistic hydrolysis of corn starch at 40, 50, and 60°C. Given the thermolability of glucoamylases, temperatures >60°C were not tested. The effect of these combinations on corn starch hydrolysis at 40°C is shown in Fig. 1A. The combination of *B. licheniformis* and *Rhizopus* mold (VIII) gives the higher yield, presenting 64% substrate conversion after 5 hr of reaction, whereas the lower starch conversions (19 and 21%) were observed with combinations III and V, respectively. All the other combinations gave intermediate yields. A difference was observed with combinations I–VI between the enzymic couples employing glucoamylase from *A. niger* and those employing *Rhizopus* mold, with the latter exhibiting about twofold higher reaction yield. This is in accordance with previous reports of increased activity of *Rhizopus* mold glucoamylase toward raw corn starch compared with *A. niger* (Yamamoto 1995). This difference also was noticed with combinations of *B. licheniformis* α-amylase, but the increase in reaction yield was smaller.

Similar results were obtained at 50°C with the same enzymic combinations (Fig. 1B). Combination VIII also gives the higher conversion of starch (76%), whereas combinations III, V, and I give only 20, 21, and 21% starch conversion, respectively. The difference observed at 40°C with the combinations of *A. niger* (I, III, V, and VII) and *Rhizopus* mold glucoamylase (II, IV, VI, and VIII) was also observed at 50°C.

In contrast to the results obtained at the previous temperatures, at 60°C (Fig. 1C) the combination of *B. licheniformis* and *A. niger* (VII) gives 66% substrate conversion and the *B. licheniformis* and *Rhizopus* mold combination (VIII) gives 48% substrate conversion. The other six combinations presented significantly lower substrate conversion of 14–22%.

Comparing corn starch conversion obtained after 5 hr of hydrolysis, at the temperatures studied, an increase in starch conversion from 40 to 50°C and a rather significant decrease from 50 to 60°C is observed with combinations II, IV, VI, and VIII. This behavior is attributed to *Rhizopus* mold glucoamylase, which generally requires low temperatures and is rapidly inactivated as temperature increases (Nicolov and Reilly 1991). In combinations I, III, and V, starch conversion was very low (≈10%) at all temperatures studied here, indicating that there is no temperature effect. In contrast, for combination VII with *A. niger* glucoamylase, an increasing reactivity on increase of temperature was observed.

The temperature effect upon hydrolysis of corn starch with α-amylases and glucoamylases used separately is shown in Fig. 2. *B. licheniformis* α-amylase gives higher sugar production than all the other amylases used and it is the only α-amylase that presents enhanced reaction yield on increased temperature. Furthermore, *Rhizopus* mold glucoamylase is presenting almost threefold higher reaction yield compared with *A. niger* at 40 and 50°C, with a significant loss of activity at 60°C. *A. niger* glucoamylase gives lower reaction yields compared with *Rhizopus* mold glucoamylase, which increase on elevation of temperature. In general, the behavior of the single enzyme system toward temperature elevation further explains the reaction profiles shown in Fig. 1.

Michaelis-Menten kinetics were determined at 50°C for each enzyme separately (Table II). The V_{max} values are of the same order for all the enzymes used, whereas K_M values reflect the differences among them. *Rhizopus* mold glucoamylase presents significantly low K_M value (0.23 g/mL), thus explaining the increased reactivity at this temperature, with *B. licheniformis* α-amylase giving the second lowest K_M value (0.38 g/mL).

TABLE I
Enzymic Combinations Used for Synergistic Hydrolysis of Corn Starch

Combination	α-Amylase	Glucoamylase
I	Porcine pancreas	*Aspergillus niger*
II	Porcine pancreas	*Rhizopus* mold
III	*Aspergillus oryzae*	*Aspergillus niger*
IV	*Aspergillus oryzae*	*Rhizopus* mold
V	*Bacillus* spp.	*Aspergillus niger*
VI	*Bacillus* spp.	*Rhizopus* mold
VII	*Bacillus licheniformis*	*Aspergillus niger*
VIII	*Bacillus licheniformis*	*Rhizopus* mold

TABLE II
Michaelis-Menten Kinetics for Enzymes Tested (50°C)

Enzyme	V_{max} (mg/mL·min)	K_M (g/mL)
Aspergillus niger	2.83	0.51
Rhizopus mold	2.45	0.23
Porcine pancreas	3.05	0.44
A. oryzae	2.42	0.56
Bacillus licheniformis	2.53	0.38
Bacillus spp.	2.72	0.44

TABLE III
Products Obtained from Corn Starch Hydrolysis by Enzymic Combinations I–VIII at 50°C After 5 hr of Hydrolysis, Determined by HPLC

	Enzymic Combination (%)							
Carbohydrates	I	II	III	IV	V	VI	VII	VIII
Glucose	95	97.5	92	94	85	95	100	100
Maltose	3	1.5	5	3	8.5	3	…	…
Maltotriose	2	1	3	3	6.5	2	…	…

The residual enzymic activity after incubation of enzymes at 40, 50, and 60°C is illustrated in Fig. 3. Only *A. niger* glucoamylase and *B. licheniformis* α-amylase retain almost initial enzymic activity at >50°C. All the other enzymes declined in activity as the temperature increased. It is interesting that *Rhizopus* mold glucoamylase loses 50% of its activity at 50°C. In addition, this explains the increased reaction yields obtained by *A. niger* glucoamylase and *B. licheniformis* α-amylase and the lower ones obtained with the other enzymes on increase of temperature.

The hydrolysis products of each combination were also determined by HPLC. The products of the eight combinations at 50°C after 5 hr of hydrolysis are given in Table III. With combinations VII and VIII, the product is 100% glucose. With the other six combinations, glucose is still the major product (85–97.5%), with maltose and maltotriose in small percentages. The increased glucose production for combinations VII and VIII could be attributed to the different reaction mode of the α-amylases. *B. licheniformis* α-amylase is a liquefying type, producing oligosaccharides with longer chains than the other α-amylases, which are saccharifying type. The higher affinity of glucoamylases to longer carbohydrate chains might lead to increased glucose production. These data further show that when *Rhizopus* mold glucoamylase is involved, glucose production is enhanced, regardless of the kind of α-amylase used.

Values shown in Fig. 1 are the average of three different experiments and the CV among them was calculated as 2.7%. The Student *t* test was employed to show the existence of statistical differences among the combinations used. Statistical analysis of data at 40°C gave significant differences for most combinations. The *t* values obtained for 5% level of probability exhibit an interesting divergence. The differences among combinations employing the same glucoamylase are small and steady with time, whereas differences among combinations with different glucoamylases present increasing *t* values with time, implying the different reaction mode of the enzymes. Combination VII does not follow the above pattern; its *t* values are of the same order with those of *Rhizopus* mold combinations, mainly due to the high reactivity of *B. licheniformis* α-amylase. Combinations III and V show no significant differences and combination VI has very small *t* values with other *Rhizopus* mold combinations and, especially with combination II, have no significant difference at 1 and 2 hr of hydrolysis. Combination VIII, which shows the higher substrate conversion at 40°C, has signi-

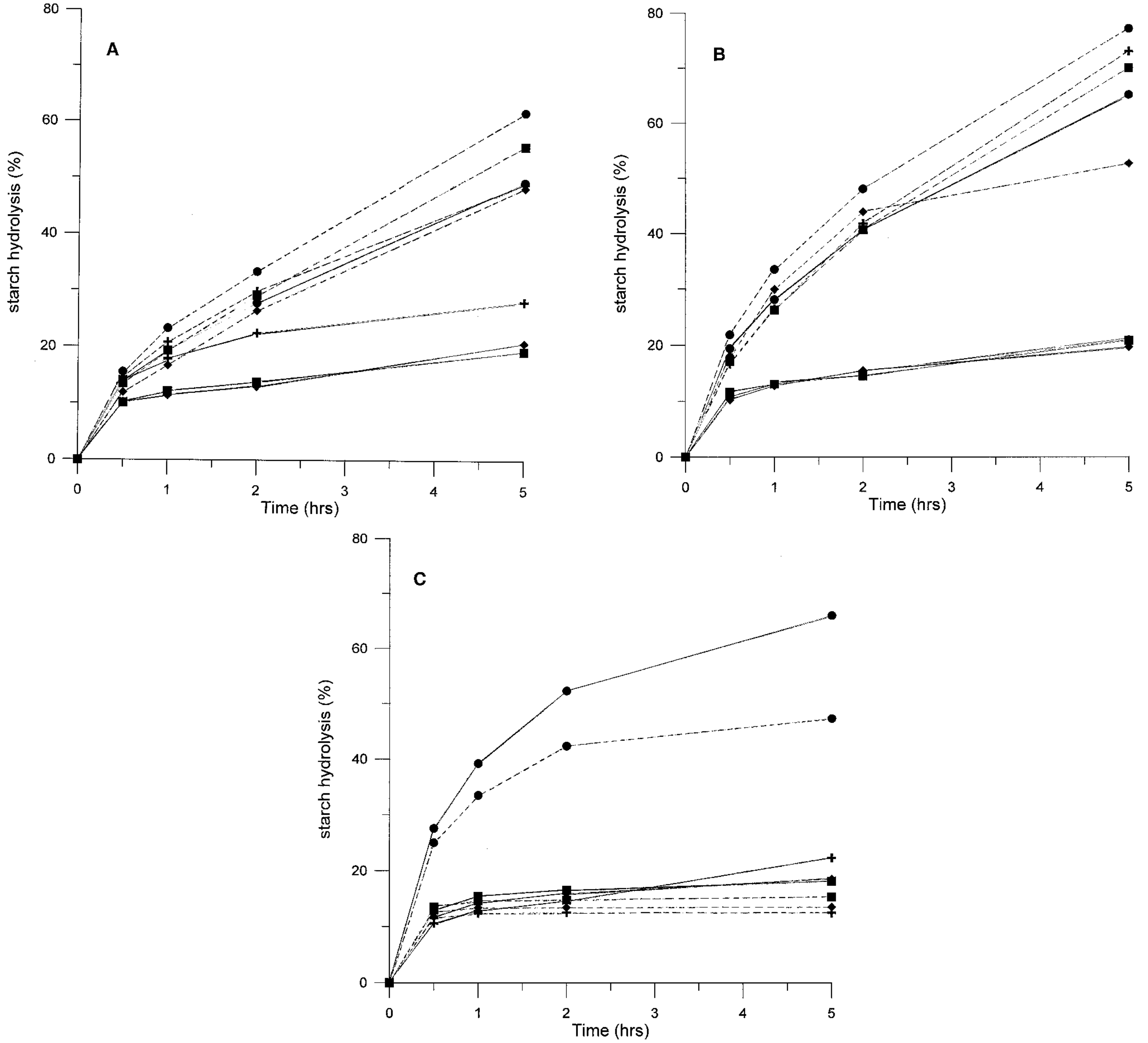

Fig. 1. Hydrolysis of corn starch at **A,** 40°C, **B,** 50°C., and **C,** 60°C by enzymic combinations. Solid lines indicate *A. niger* glucoamylase and dashed lines indicate *Rhizopus* mold glucoamylase. Combinations I and II (✚), III and IV (◆), V and VI (■), and VII and VIII (●).

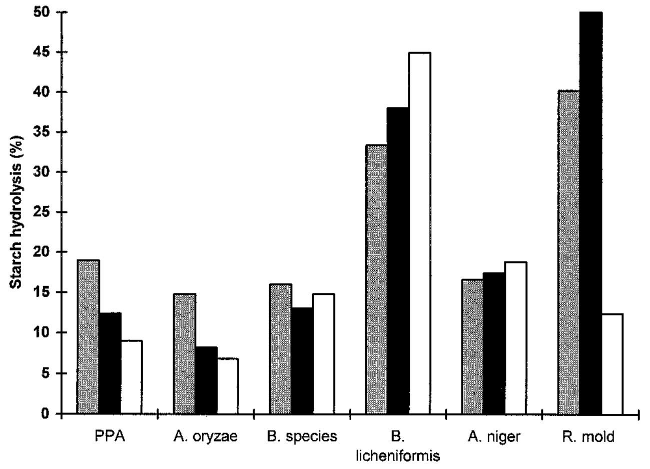

Fig. 2. Comparison of corn starch hydrolysis after 5 hr at different temperatures (40, 50, and 60°C, left to right) with a single enzyme.

ficant differences with high *t* values with all combinations, especially with those containing *A. niger* glucoamylase.

Similar results were obtained with experiments conducted at 50°C. There are significant differences with increasing *t* values as time passes for combinations with different glucoamylases. Again, combination VII gives small *t* values with all *Rhizopus* mold combinations, with no significant differences for some values. Concerning the combinations employing the same glucoamylase, the three *A. niger* combinations (I, III, and V) have no significant difference and are practically following the same reaction course. *Rhizopus* mold combinations also present similarities with very small *t* values for reaction times up to 2 hr.

At 60°C, the apparent diversion among combinations with different glucoamylases does not exist. Combinations I–VI have very low *t* values and, in many cases, no significant difference is observed.

The only obvious differences are found with combinations of *B. licheniformis.* This is expected because their productivity is approximately three times higher than the previous combinations. Interestingly enough, the combinations of the α-amylases from PPA, *A. oryzae,* and *Bacillus* spp. have no significant difference at 0.5 and 1 hr, regardless of the glucoamylase used.

CONCLUSIONS

It is evident from the experimental data and statistical interpretation that the origin of enzymes can affect corn starch hydrolysis. Combinations of α-amylases with *Rhizopus* mold glucoamylase can be used for hydrolysis of corn starch at 40 and 50°C, giving two to three times higher yield than those containing glucoamylase from *A. niger*. At 60°C, *A. niger* glucoamylase combinations can be used because *Rhizopus* mold glucoamylase is rapidly inactivated. Concerning α-amylases, combinations employing *B. licheniformis* were the most efficient at all temperatures studied. The other α-amylases give comparably lower yields and lose their activity rapidly with increase of temperature. The experiments showed that *A. niger* glucoamylase and *B. licheniformis* α-amylase were the only enzymes that retained their initial activity after incubation at the temperatures studied. The synergistic hydrolysis of crude corn starch presents $\leq$76% starch conversion in only 5 hr of reaction, without prior gelatinization of starch, thus saving energy costs. It also favors glucose production over other oligosaccharides, leading to almost 100% glucose production.

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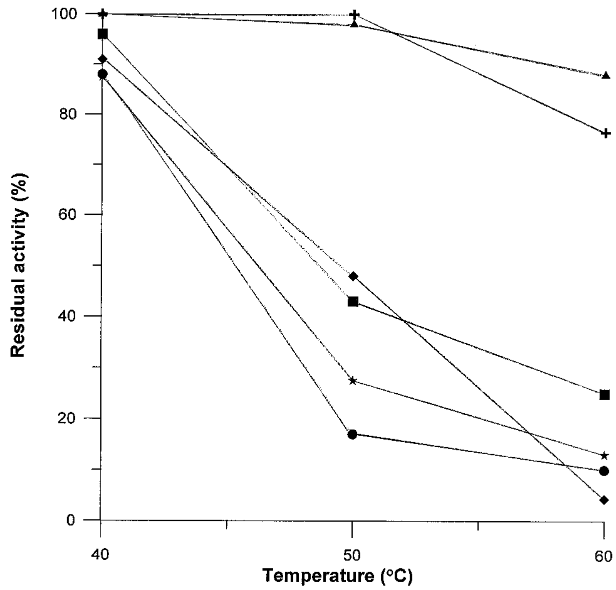

Fig. 3. Residual enzymic activity after 2 hr of incubation of the enzymes at 40, 50, and 60°C. *Aspergillus niger* (✚), *Rhizopus* mold (◆), porcine pancreas (■), *A. oryzae* (●), *Bacillus licheniformis* (▲), and *Bacillus* spp. (★).

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