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Enzymatic Hydrolysis of Chestnut Purée: Process Optimization Using Mixtures of α-Amylase and Glucoamylase

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The enzymatic hydrolysis of starch present in chestnut purée was performed through a one-step treatment with a mixture of a commercial thermostable α-amylase (Termamyl 120L, type S) and glucoamylase (AMG 300L) at 70 °C. The effect of the enzyme concentration and the ratio of both amylases in the reaction mixture was studied by means of a factorial second-order rotatable design, which allowed conditions to be set leading to the total conversion of starch to glucose after 15 min of incubation (60 total enzymatic units g^{-1} of chestnut; ratio of α-amylase/glucoamylase enzymatic units, 0.35:0.65). At lower enzyme concentration, the delay in the addition of the glucoamylase with regard to the addition of the α-amylase allowed a slightly higher hydrolysis percentage to be reached when compared to the simultaneous addition of both amylases at the same low enzyme concentration. The kinetics of liberation of glucose supports the existence of a synergistic effect between these two enzymes only in the first moments of the reaction. Finally, a sequential one-step hydrolysis was assayed, and more concentrated glucose syrups were thus obtained.

KEYWORDS: Chestnut starch; one-step hydrolysis; glucoamylase; α-amylase; synergistic effect

INTRODUCTION

Corn, wheat, rice, and potato are the main sources of starch in the world for industrial purposes (especially sugar and alcohol manufacture). Nevertheless, several indigenous crops can constitute local alternative sources of starches and lead to conservation of agriculturally marginal lands (*1*). In this sense, the cultivation of the chestnut tree in internal areas of Galicia (northwestern Spain) and northern Portugal dates from the first century, constituting ∼20% of the European production of chestnuts. Besides the use of the crop for human seasonal fresh consumption, only a minimum part of chestnuts is manufactured in the confectionery industry (mainly to elaborate *marron glace*), whereas an important amount of the total production is destined for local animal feed. One way to improve the value of overproduction and the bad-quality chestnut could be the manufacture of new products such as a distilled spirit obtained from postincubates of alcoholic fermentation of chestnut with yeast. The first step of this alternative requires the conversion of starch into fermentable sugars.

Generally, enzymatic conversion of starch into glucose syrup involves gelatinization, liquefaction with α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1.), and saccharification with glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) of the maltodextrins previously formed (*2*). At industrial scale, the hydrolysis of starch is usually carried out in two steps, consisting in a first stage of simultaneous gelatinization and liquefaction with a high-temperature α-amylase and a second one of saccharification.

Depending on the plant, the tissue, and the crystalline structure of the starch granules, the composition, viscosity, and degradation resistance of starches vary. Research in the liquefaction process has been focused to find, in each case, suitable conditions for gelatinization (intensity, extruders), in addition to adequate conditions of reaction (pH, temperature, enzyme/substrate ratio) with thermostable enzymes (*3*). With regard to the saccharification step, efforts have been directed to the prevention of inhibition and reversion reactions to increase the yield in glucose syrups (*4, 5*).

On the other hand, studies on process optimization of hydrolysis have been usually performed by varying one variable while keeping the other ones at a constant level and, consequently, neither true optimum conditions nor interactions between variables can be detected. The use of factorial experimental designs avoids these inconveniences through the obtaining of empirical models, which describe the effect of the variables considered on the process. These approaches have been successfully applied for optimization of saccharification of mussel processing wastes (*6*) and liquefaction of sago (*3*) and potato starch (*7*).

When liquefaction and saccharification are separated processes, relatively long reaction times are needed. To reduce them, the use of mixtures of enzymes in a single step has been proposed on the basis of the synergistic mode of action of α-amylase and glucoamylase in the hydrolysis of starch (*8*). A

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Table 1. Composition of Raw Chestnut without Teguments

component	g/100 g (wet basis)
water content	56.9 ± 1.0
total sugars	36.7 ± 0.8
sucrose	6.5 ± 0.1
starch	30.2 ± 0.8
glucose	traces
total nitrogen	0.46 ± 0.02
proteins	2.24 ± 0.07
total phosphorus	0.052 ± 0.002
lipids	1.70 ± 0.05
fiber	1.21 ± 0.07
ash	1.02 ± 0.03

rapid conversion of starch into glucose is particularly interesting to avoid substrate limitation in those cases when both hydrolysis and fermentation are simultaneously performed. Nevertheless, antagonistic effects have been suggested due to the different rates at which α-amylase generates, or fails to generate, optimum molecular size substrates for the action of glucoamylase (*9*).

To valorize chestnut as a source of glucose for different fermentation purposes (including the production of ethanol to obtain a distilled spirit), the present paper studies the optimization of a simultaneous liquefaction−saccharification process of chestnut starch using mixtures of thermostable α-amylase and glucoamylase.

MATERIALS AND METHODS

Substrate. The composition of the peeled raw chestnut used in this work is shown in **Table 1**. The starch content of raw chestnut was calculated from the difference between total sugars (cellulose and pentosanes are not included) and sucrose content, considering no other sugars were found in measurable amounts.

Enzymes and Hydrolysis Conditions. The enzymes used in this work and the conditions for the preliminary assays of hydrolysis, fixed according to the recommendations of the manufacturer (Novo Nordisk A/S Industries, Bagsvaerd, Denmark), are shown in **Table 2**.

Raw chestnuts, slightly steamed to remove external teguments, were chopped and suspended in suitable buffers containing 12 ppm of $CaCl_2$ to stabilize the α-amylase. This suspension was gelatinized by heating in autoclave for 1 h at 100 °C for simultaneous sterilization in view of the use of the hydrolysate as susbstrate for fermentation. After hot homogenization (3 min in an Ultraturrax at 9500 rpm), the suspension (purée) was immediately used as substrate (or maintained at 70 °C) to avoid retrogradation.

The hydrolysis was carried out at controlled temperature in a thermostated bath, under orbital agitation, in sealed 50 mL Erlenmeyer flasks loaded with 15 mL of chestnut suspension (chestnut purée). The specific conditions of each assay will be described in connection with the corresponding results. At fixed times, the reaction was stopped by the addition of 10 N NaOH (never more than 1% of total volume; resulting pH of ∼12). After sample centrifugation (12000*g*), the supernatants were recovered for analytical determinations.

Analytical Methods. Total sugars (TS) were measured according to the phenol−sulfuric method of Dubois et al. (*10*) using glucose as standard. Total sugars in raw chestnut (cellulose and pentosanes are not considered) were determined as described above after complete solubilization. This was achieved by enzymatic treatment of a 25 g L^{-1} (wet basis) chestnut suspension, buffered at pH 6.0 and incubated at 85 °C for 2 h, with a high-temperature α-amylase [Termamyl 120L (L)] at a concentration of 200 EU g^{-1} of raw chestnut (wet basis). Mono-, di-, and oligosaccharides were analyzed by reverse-phase HPLC with a refractive index detector, according to the method of Franco and Garrido (*11*). The column was a Spherisorb R ODS2 (25 cm × 0.46 cm) from Waters operated at room temperature, with water as mobile phase in isocratic conditions, adjusting the pH of samples in the range of 6−7. Mono- and disaccharides in raw chestnuts were previously extracted as described by AOAC (982.14) (*12*). Reducing sugars (RS) were determined by the 3,5-dinitrosalicylic acid (DNS) reaction (*13*) with glucose as standard. Amylolytic activity (AA) was measured in enzymatic units (EU) as described by Murado et al. (*14*). Additional analytical methods were as follows: proteins (*15*), total nitrogen (*16*), total phosphorus (*17*) (after hot acid mineralization), fiber (cellulose) by AOAC (962.09) (*18*), and total lipids by ISO R:1433 norm (*19*). All analytical determinations were made in duplicate.

Criteria for Hydrolysis Evaluation. To evaluate the progress of the enzymatic reaction, the following criteria were defined (expressed as percentage): *solubilization* (*S*), defined as the ratio between TS in the supernatant obtained from the mixture of reaction and TS (excepting cellulose and pentosanes) in the chestnut purée, and *hydrolysis* (*H*), defined as the ratio between RS in the supernatant obtained from the mixture of reaction and TS (excepting cellulose, pentosanes, and sucrose) in the chestnut purée. According to this definition and the sugar composition of chestnut, 100% of hydrolysis corresponds with total conversion of starch to glucose.

Statistical Methods. The simultaneous effect on chestnut purée hydrolysis of the *enzyme concentration* (*E*) and the ratio between α-amylase and glucoamylase, expressed as the *ratio of* α*-amylase/glucoamylase* enzymatic units referred to one total enzymatic unit (*R*), was studied by means of a second-order rotatable experimental plan with $\alpha = 1.267$ and five replicates in the center of the domain, according to Akhnazarova and Kafarov (*20*) and Box et al. (*21*). The e*nzyme concentration* (*E*) was defined as the sum of the individual AA of each enzyme alone and expressed as EU g^{-1} of chestnut (wet basis). Percentages of *solubilization* (*S*) and *hydrolysis* (*H*), as well as glucose generation from starch, were used as dependent variables to evaluate the process. Experimental domain and coding criteria are given in **Table 3**. Significance of the coefficients of the models was calculated using Student's *t* test ($\alpha < 0.05$) as acceptance criterion. The global consistency of the models was verified by Fisher's *F* test ($\alpha < 0.05$) applied to total error/experimental error and lack of fitting/experimental error quotients.

RESULTS AND DISCUSSION

Selection of the Enzymes. To select an α-amylase and a glucoamylase for further experiments, the ability of several commercial amylases to hydrolyze the starch present in chestnut purée was evaluated. For this purpose individual kinetics of

Table 2. Main Characteristics and Reaction Conditions for the Commercial Liquid Amylases, Purchased from Novo, Assayed for the Hydrolysis of Starch Present in Chestnut Purée

enzyme	type[a]	microorganism[b]	AA[c] (EU mL^{-1})	*T* (°C)	pH	buffer
AMG 300L	G	*A. niger*	2459	70	4.5	citric–phosphate, 50 mM
SAN SUPER 240L	G	*A. niger*	6618	55	5.5	citric–phosphate, 50 mM
BAN 240L	α	*B. amyloliquefaciens*	15327	70	7.0	phosphate, 50 mM
Termamyl 120L (L)	α	*B. licheniformis*	3878	85	5.7	citric–phosphate, 50 mM
Termamyl 120L (S)	α	*B. licheniformis*[d]	7340	70	5.7	citric–phosphate, 50 mM
Fungamyl 800L	α	*Aspergillus* sp.	44339	40	5.7	citric–phosphate, 50 mM

[a] G, glucoamylase; α, α-amylase. [b] *A.*, *Aspergillus*; *B.*, *Bacillus*. [c] AA, amylolytic activity expressed in enzymatic units (EU) mL^{-1} as described by Murado et al. (*14*). [d] *B. licheniformis* expressing *B. stearothermophilus* α-amylase.

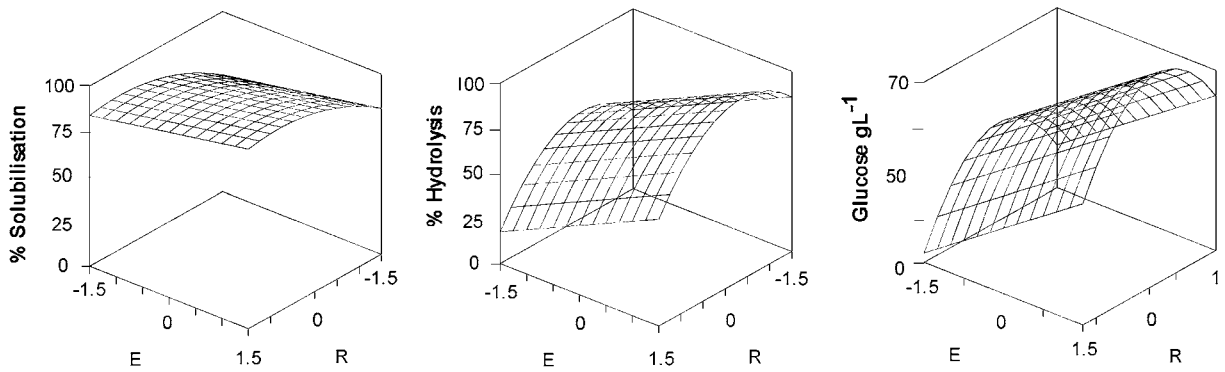

Figure 1. Response surfaces showing the effect of the enzyme concentration (*E*) and the ratio of α-amylase/glucoamylase enzymatic units (*R*) on the solubilization, hydrolysis, and glucose release from chestnut purée, according to eqs 1, 2, and 3, respectively (**Table 4**).

Table 3. Experimental Domain and Codification of the Independent Variables Analyzed by Means of a Second-Order Rotatable Factorial Design Applied to the Study of the Enzymatic Solubilization, Hydrolysis, and Glucose Generation from Chestnut Purée

	natural values	
coded values	E[a] (EU g^{-1})	R[b]
-α (–1.267)	6.0	0
–1	12.0	0.105
0	33.5	0.5
+1	54.0	0.895
+α (+1.267)	60.0	1
codification	V_n = natural value	
$V_c = (V_n - V_0)/\Delta V_n$	V_c = coded value	
decodification	V_0 = natural value in the center of the domain	
$V_n = V_0 + (\Delta V_n V_c)$	ΔV_n = increment of V_n corresponding to 1 unit of V_c	

[a] *E*, enzyme concentration expressed as enzymatic units (EU) g^{-1} (raw substrate). [b] *R*, ratio of α-amylase/glucoamylase enzymatic units, referred to 1 total enzymatic unit in the mixture.

hydrolysis of 40 g L^{-1} chestnut purée were carried out, keeping each enzyme in the conditions of pH and temperature referred to in **Table 2**. The amount of each enzyme was adjusted to provide a final concentration in the mixture of 50 EU g^{-1} of chestnut (wet basis).

Total solubilization or hydrolysis was not achieved in any case (data not shown). The kinetic behavior of the enzymes segregated according to their mode of action and the evaluation criteria used. Thus, the best results of solubilization corresponded to α-amylases Termamyl 120L (S) and 120L (L), whereas AMG 300L and San Super 240L yielded the highest values of hydrolysis. As the differences obtained between the two best α-amylases and between the glucoamylases were small, an α-amylase and a glucoamylase with similar conditions of operation were selected for the next experiments. With this criterion Termamyl 120L (S) and AMG 300L were chosen.

Optimization of the Hydrolysis of Chestnut Purée Using a Combination of Termamyl 120L (S) and AMG 300L. Due to the synergistic mode of action of α-amylase and glucoamylase proposed by several authors (*8*), the use of mixtures of both types of enzymes in a single step to completely hydrolyze starch appears to be a good alternative to the more habitual process in two consecutive steps of liquefaction and saccharification.

The effect of the enzyme concentration (*E*) and the ratio of α-amylase/glucoamylase enzymatic units (*R*) on the percentages of solubilization and hydrolysis of starch contained in chestnut purée was studied by means of a second-order rotatable two-level factorial design (*20*). Even though the effect of the enzyme concentration on the hydrolysis does not generally follow linear or parabolic patterns, these can be accepted for a narrow range of this independent variable. The reaction conditions were as follows: temperature of incubation, 70 °C; concentration of chestnut purée, 225 g L^{-1}; initial pH, 4.75 (value of compromise for both enzymes); and time of reaction, 15 min.

The empirical models obtained for the percentages of solubilization and hydrolysis (**Table** 4) showed good fittings. In the assayed range, *E* influenced hydrolysis and solubilization in a linear mode (**Figure 1**), leading to better responses as this variable increases, without interactions with *R*. Contrarily, *R* presented a second-order term in both models for solubilization and hydrolysis, as well as a first-order term. The second-order terms implied the existence of optimum values of *R* inside the experimental domain (which means mixtures of both enzymes), which led to total solubilization and hydrolysis in combination with the highest value of *E* assayed (**Table 4**), this indicating

Table 4. Empirical Models for the Percentages of Solubilization (*S*), Hydrolysis (*H*) and Glucose Concentration (*G*) of Hydrolysates from Chestnut Purée

		coefficients of the model[b]										
eq	Y[a]	i.t.	E	R	$E \times R$	E^2	R^2	r^2 [c]	r^2_{adj}[c]	E_{opt}[d]	R_{opt}[d]	Y_{max}[e]
1	% *S*	90.4	5.4	6.2	NS	NS	–3.6	0.731	0.641	+1.267 (60 EU g^{-1})	+0.876 (0.846)	99.9
2	% *H*	78.1	13.9	–8.8	NS	NS	–11.7	0.848	0.798	+ 1.267 (60 EU g^{-1})	–0.377 (0.351)	97.3
3	*G* (g L^{-1})	50.3	13.0	–14.1	NS	NS	–14.2	0.925	0.900	+ 1.267 (60 EU g^{-1})	–0.495 (0.304)	70.3

[a] Response. [b] Coefficients for the terms of the model (i.t., independent term; *E*, enzyme concentration (EU g^{-1}); *R*, ratio of α-amylase/glucoamylase enzymatic units). [c] Regression coefficients (adj, adjusted). [d] Optimal values of *E* and *R*: coded values and (natural values). [e] Maximum response for the optimal values of the independent variables.

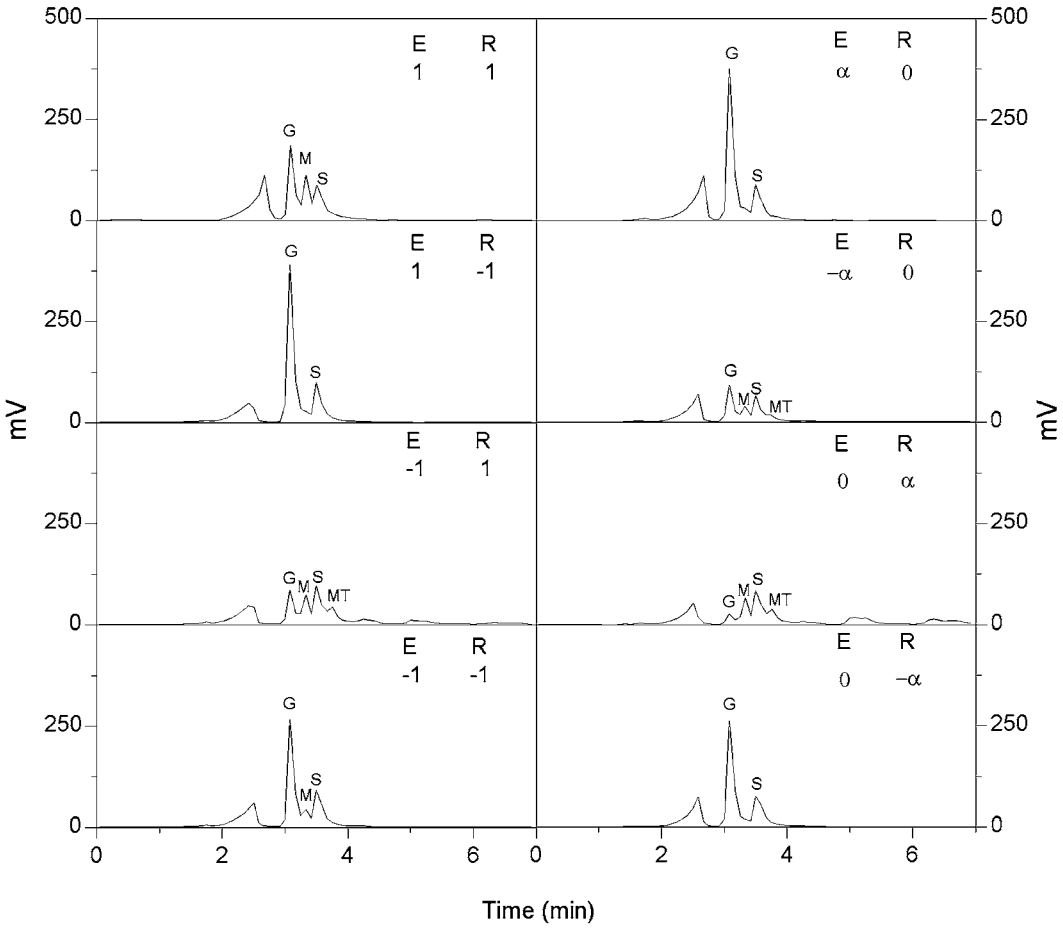

Figure 2. Sugar composition of the chestnut hydrolysates corresponding to different combinations of *E* and *R* assayed in the rotatable factorial design: glucose (G), maltose (M), saccharose (S), and maltotriose (MT).

the convenience of both enzymes working together. In any case, the opposite sign of the first-order *R* term coefficients in models 1 and 2 (**Table 4**) reflects the weight of each enzyme in each process affecting the optimum values of *R*. In fact, the solubilization was favored when α-amylase was the main enzyme in the mixture of reaction (positive sign of the first-order *R* term coefficient in model 1; optimum coded value, $R = +0.795$; natural value = 0.814), whereas a high ratio of glucoamylase was necessary to maximize the percentage of hydrolysis (negative sign of the first-order *R* term coefficient in model 2; optimum coded value, $R = -0.377$; natural value = 0.351).

Because hydrolysis implies solubilization, the first term was used as the only criterion for the next assays.

Sugar Composition of the Hydrolysates. From a practical point of view, the percentages of solubilization and hydrolysis in the above experiments were suitable indicators and provided significant information about the effect of *E* and *R* (in the assayed range) on the hydrolysis of starch in chestnut purée. Nevertheless, it is possible that different qualities of sugars correspond to the same value of percentages of solubilization or hydrolysis. This eventuality suggests that modification of the variables of the system (*E* and *R* as well as pH and temperature) could permit the design of suitable sugar profiles in the mixture of reaction (*7*, *21*) for different purposes (e.g., alcoholic fermentation).

The sugar profiles corresponding to the combinations of the variables assayed in the previous factorial experiment were different depending on the values of *E* and *R* (**Figure 2**). Sucrose, maltose, and glucose were the main sugars in the hydrolysates. The levels of sucrose remained invariable for all of the conditions because of the inactivation of the endogenous invertase and sucrose synthase during the gelatinization step at 100 °C. Glucose was the only product of starch hydrolysis present in all of the situations. Maltotriose and oligosccharides of higher degree of polymerization were detected in only those cases total or partially lacking in glucoamylase. For maltose the situation was quite similar but less extreme. The negative effect on maltose release of the increase of the ratio of glucoamylase in the mixture can be partially overcome by decreasing the total enzyme concentration (compare: $E = 1$; $R = -1$ without maltose; and $E = -1$; $R = -1$).

To describe the composition of the hydrolysates for different combinations of the variables *E* and *R* inside the experimental domain, an empirical model for glucose concentration was obtained (**Table 4**). As expected, *E* and *R* showed similar behaviors in models 3 for glucose and 2 for hydrolysis, predicting total conversion of starch to glucose at approximately the same optimum value of *R*, corresponding to a mixture rich in glucoamylase. Despite the reduced contribution of the α-amylase to the release of glucose from starch, the need of a minimal amount of this enzyme to optimize the production of the monosaccharide points to a synergistic effect between these two enzymes.

Hydrolysis with a Mixture of Amylases at Low Enzyme Concentration. Total hydrolysis of starch present in chestnut purée in a short time of reaction was possible using α-amylase−glucoamylase mixtures in an adequate proportion and with a high enzyme/substrate ratio. Nevertheless, from an economical point of view, the cost of enzymes could represent a limiting

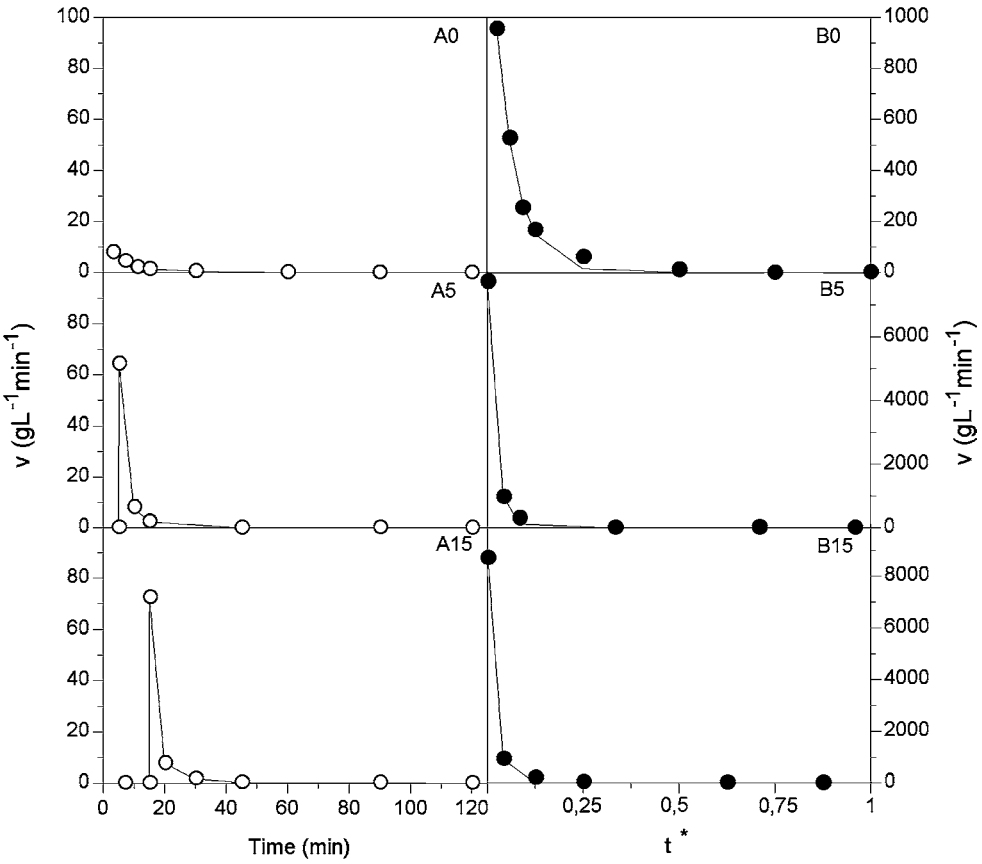

Figure 3. (**A***i*) Experimental rates (open symbols) of glucose production from chestnut purée referred to real times of reaction (t_r; Time); (**B***i*) experimental values (solid symbols) and modeling (continuous line; eq 5) of glucose production rates referred to adimensional times of reaction (t^*). *i* is the time of retarded addition of glucoamylase.

factor for an industrial process. For this reason, it is reasonable to suppose the feasibility of reaching total hydrolysis by decreasing the total concentration of the enzyme and extending the time of incubation. To check this possibility, a kinetic study of the one-step hydrolysis of a 225 g L^{-1} chestnut purée was performed at the lowest value of enzyme concentration tested, keeping R in the optimum value for hydrolysis ($R = -0.377$, coded value).

The results (data not shown) showed the inability of this low enzyme concentration to get the theoretical maximum level of starch conversion. In fact, after 72 h of incubation, the percentage of hydrolysis reached an asymptote of 89%. As this behavior cannot be attributed to the existence of a limit dextrin (total hydrolysis of the starch was yet achieved at the highest concentration of enzymes), phenomena such as product or substrate inhibition and/or thermal deactivation could explain the stoppage of the reaction in these conditions before total hydrolysis. In fact, it is known that glucoamylase presents substrate (*9*) and product inhibition (*22*), as well as high susceptibility to thermal deactivation (*23*−*25*), these effects being more intense when the enzyme/substrate ratio is low. Studies of inhibition and thermal deactivation will be related in following papers.

Retarded Addition of Glucoamylase. An alternative to reduce the possible effects of substrate inhibition and thermal deactivation operating at low enzyme concentration could be the slight delay of the addition of the glucoamylase with respect to the addition of the α-amylase, before the complete reaction of this last enzyme on starch. This approach would make compatible the use of the enzyme mixture in a sole step with the previous reduction of the viscosity of the medium (responsible for glucoamylase substrate inhibition in some cases) by the partial action of the α-amylase. Additionally, the diminution of the glucoamylase exposure time to high temperatures would allow the thermal inactivation of this enzyme to be reduced.

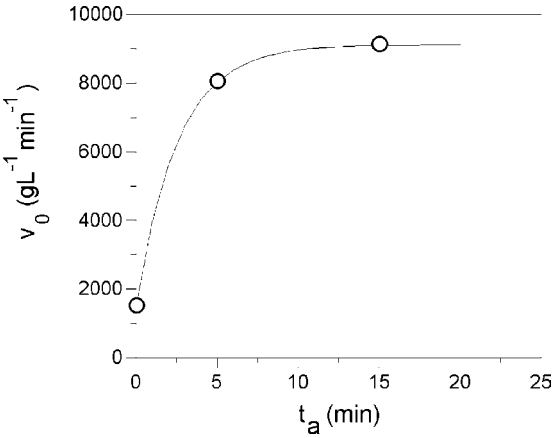

Figure 4. Rates of glucose production at the moment of addition of glucoamylase (v_0), previously calculated from eq 5 (symbols) and adjusted according to eq 6 (continuous line) for different times of retarded addition of glucoamylase (t_a).

To confirm this hypothesis, glucoamylase was added to the mixture of reaction simultaneously to α-amylase and at 5 and 15 min after the incorporation of this last enzyme. Because the percentage of hydrolysis is a criterion less sensitive to inhibition phenomena than the rate of glucose liberation, this was additionally considered to evaluate the suitability of this approach.

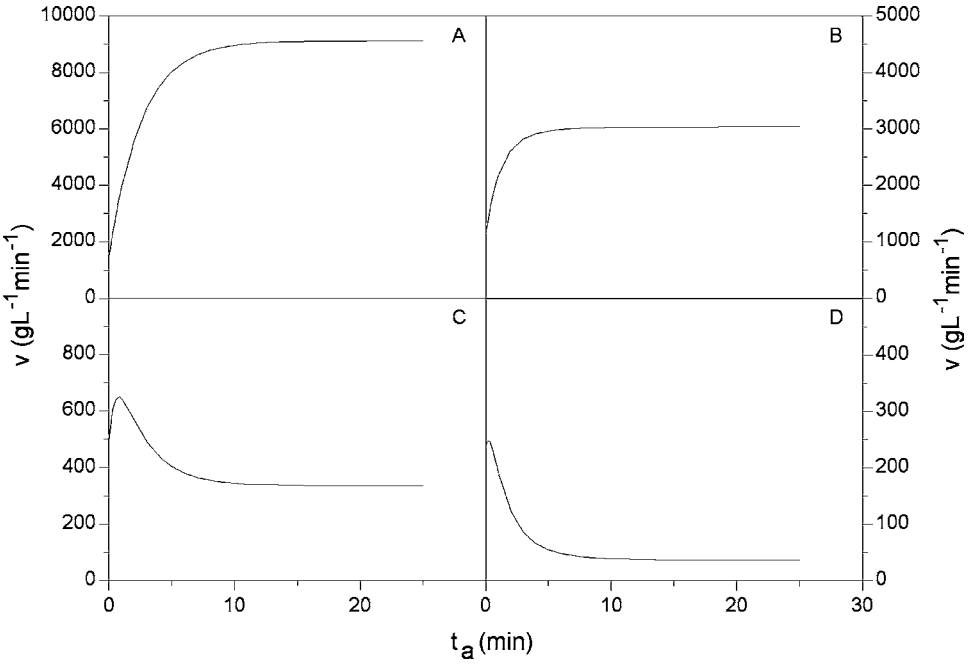

Figure 5. Simulation of the rates of glucose production for different times of retarded addition of glucoamylase (t_a) at four different adimensional times: $t^* = 0$ (**A**), $t^* = 0.02$ (**B**), $t^* = 0.06$ (**C**), and $t^* = 0.1$ (**D**), according to the combination of eqs 5 and 6.

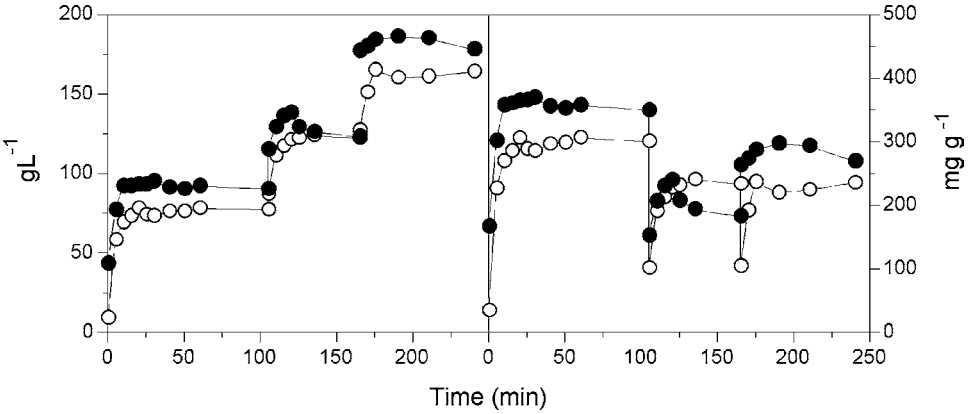

Figure 6. Subsequent cycles of one-step hydrolysis of chestnut purée performed at the optimal conditions predicted by eq 2. Evolution of the concentration of TS (solid symbols) and RS (open symbols), expressed as g L^{-1} in the supernatant and as mg g^{-1} of substrate, respectively.

In fact, the series with a delay of 15 min in the addition of glucoamylase yielded a slightly higher maximum percentage of hydrolysis than the series with simultaneous or closer addition of both enzymes (data not shown), although it was not possible in any case to reach total hydrolysis. The improvement obtained by delaying glucoamylase addition was more noticeable when the rate of glucose generation was considered (**Figure 3A*i***). The maximum rate of glucose release obtained for the longest retardation of glucoamylase addition was 8-fold higher than for joint addition. Because the viscosity of the chestnut purée decreased sharply just in the moment of α-amylase addition, thus avoiding diffusional restrictions for glucoamylase in the series corresponding to 5 and 15 min of delay of glucoamylase addition, these results appear to mean that the decrease in the molecular weight of the oligosaccharides produced by the previous action of the α-amylase during the first 15 min of reaction favored the subsequent action of the glucoamylase.

Unfortunately, the determination of the optimum size of the substrate for the action of glucoamylase has some experimental difficulties relating to the availability of well-characterized distributions of molecular weights of dextrins generated after the attack of starch by the α-amylase. Nevertheless, mathematical simulation of the hydrolytic process in these systems can constitute a useful tool to estimate the role of the products generated by the α-amylase in the behavior of the glucoamylase.

Table 5. Values of the Parameters According to Equation 6

parameter	value
$v_0{}^{o\,a}$	1486
$v_{0,max}{}^{b}$	9126
m^c	0.389

[a] Rate of glucose generation at the moment of addition of glucoamylase. [b] Highest rate of glucose generation. [c] Exponential parameter of adjustment.

To build an algorithm to simulate the rate of glucose production when glucoamylase was added with delay to α-amylase to the mixture of reaction, the following assumptions were made:

(1) Because of the differences in the time of reaction generated by the delay in the addition of glucoamylase, it is difficult to compare the action of both enzymes working together at the same time of incubation. For this reason, it was necessary

to equalize the times of reaction according to

$$t^* = (t_r - t_a)/t_t \quad (4)$$

where t^* corresponds to an adimensional time at which both enzymes work together, t_r is the real time of reaction since the addition of α-amylase, t_a is the time at which the glucoamylase is added, and t_t is the total time of the reaction.

(2) The kinetics of the rate of glucose generation can be phenomenologically described in each case by first-order kinetics (**Figures 3B*i***; eq 5)

$$v = v_0 \, e^{-kt^*} \quad (5)$$

where v is the rate of glucose production referred to t^*, v_0 represents the rate of glucose production at the time of addition of glucoamylase ($t^* = 0$) and the maximum rate for each time of addition (t_a), and k is a first-order kinetic constant. Both v_0 and k depend on the time of addition (t_a).

(3) When representing v_0 versus t_a, an asymptotic relation was observed (**Figure 4**), which can be described by eq 6. It allows predicting the maximum rate of glucose formation for different times of addition of glucoamylase.

$$v_0 = v_{0,max} + (v_0^{\,0} - v_{0,max}) \, e^{-mt_a} \quad (6)$$

$v_0^{\,0}$ is the rate of glucose production at the moment of addition of glucoamylase ($t^* = 0$) when $t_a = 0$, $v_{0,max}$ is the highest rate of glucose production, and m is a parameter of adjustment (**Table 5**).

(4) By combining eqs 5 and 6 it was possible to simulate the rate of glucose production referred to t^* for different retarded times of addition of glucoamylase (**Figure 5**).

From the estimations obtained by simulation, it can be concluded that at the moment of the addition of glucoamylase ($t^* = 0$), the rate of glucose formation increases in an asymptotic way when t_a increases due to the diminution of the molecular weight of the substrate as a consequence of the longest time of the α-amylase reaction (**Figure 5A**). As the time of reaction advances (t^*), the rate of glucose production for different t_a decreases and changes from an increasing asymptotic pattern (**Figure 5A**,**B**) to a descendent asymptotic behavior, which shows an initial maximum that moves to smaller values of t_a as much as the time of reaction increases (**Figure 5C**,**D**).

According to Fujii and Kawamura (*8*), this simulation model corroborates that there is an optimal molecular weight oligosaccharide for the action of glucoamylase, which is provided by the previous action of the α-amylase on starch. This indicates the existence of a synergistic effect between glucoamylase and α-amylase when using starch as substrate, which would occur only in the first moments of the reaction with both enzymes working together.

One-Step Hydrolysis in Subsequent Cycles. Two main factors prevent the achievement of high-glucose concentrates from chestnut: first, the low concentration of starch in raw chestnut and, second, the impossibility of working with chestnut purée concentrations >300 g L^{-1} because of their viscosity.

A procedure to increase the glucose concentration in chestnut hydrolysates could consist of consecutive cycles of one-step hydrolysis developed using the hydrolysate obtained in the first cycle, free of solids, to make a new chestnut purée for the next cycle of hydrolysis. To test the suitability of this approach, an experiment was performed in which three consecutive stages of one-step hydrolysis were carried out in the conditions previously optimized (E = 60 EU g^{-1}; R = 0.351, natural values). In this case chestnut purée concentration was raised to 250 g L^{-1} and the enzymes were added at the beginning of every cycle in the optimal concentration and ratio.

The results (**Figure 6**) confirmed the ability of the procedure to reach a higher concentration of total and reducing sugars in the hydrolysate than in the simple process (a sole cycle of one-step hydrolysis). Nevertheless, there is a strong decrease of the efficiency of the hydrolysis at the second cycle (**Figure 6**), probably due to an effect of product inhibition, as was previously indicated. Although it would be reasonable to expect a gradual decrease of the hydrolyzing ability as long as glucose accumulates in the supernatant, this does not happen in the last stage, where the efficiency in reducing and total sugars is comparable to, or even slightly higher than, the efficiency in the precedent stage. This behavior probably has to do with the residual amylolytic activity detected at the end of every cycle, which joins the amylolytic activity due to the addition of fresh enzymes at the beginning of the next cycle. This way, the initial amylolytic activity at the second and third cycles increased 39 and 92%, respectively, with regard to the first one (60 EU g^{-1}). Considering the relatively low increase of the product concentration in the hydrolysate after the second cycle with respect to the first one, the higher initial amylolytic activity of the last stage means a higher enzyme/products ratio, which contributes to reduce the probable inhibitor effect.

The higher improvement of total sugar efficiency compared with reducing sugars efficiency in the last cycle could be attributed to a gradual enrichment of the enzymatic mixture in α-amylase with regard to the glucoamylase as a consequence of a possibly higher stability of the α-amylase. This hypothesis as well as the existence of inhibition phenomena will be studied in subsequent papers.

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