FEBS Letters 452 (1999) 346–350 FEBS 22124

Alcoholysis reactions from starch with α-amylases

R.I. Santamaría, G. Del Río, G. Saab, M.E. Rodríguez, X. Soberón, A. López-Munguía*

Instituto de Biotecnología, UNAM, Apartado Postal 510-3, Cuernavaca, Morelos 62271, Mexico

Received 1 March 1999; received in revised form 29 April 1999

Abstract The ability of α-amylases from different sources to carry out reactions of alcoholysis was studied using methanol as substrate. It was found that while the enzymes from *Aspergillus niger* and *Aspergillus oryzae*, two well-studied saccharifying amylases, are capable of alcoholysis reactions, the classical bacterial liquefying α-amylases from *Bacillus licheniformis* and *Bacillus stearothermophilus* are not. The effect of starch and methanol concentration, temperature and pH on the synthesis of glucosides with α-amylase from *A. niger* was studied. Although methanol may inactivate α-amylase, a 90% substrate relative conversion can be obtained in 20% methanol at a high starch concentration (15% w/v) due to a stabilizing effect of starch on the enzyme. As the products of alcoholysis are a series of methyl-oligosaccharides, from methyl-glucoside to methyl-hexomaltoside, alcoholysis was indirectly quantified by high performance liquid chromatography analysis of the total methyl-glucoside produced after the addition of glucoamylase to the α-amylase reaction products. More alcoholysis was obtained from intact soluble starch than with maltodextrins or pre-hydrolyzed starch. The biotechnological implications of using starch as substrate for the production of alkyl-glucosides is analyzed in the context of these results.

Key words: Alcoholysis; Fungal α-amylase; Transglucosidase; Saccharifying

1. Introduction

Glucosidases and glucosyltransferases constitute one of the most important groups of enzymes and have become the basis of the processes involved in glycobiotechnology. This is the case of α-amylase (E.C. 3.2.1.1), a key enzyme in the metabolism of a wide variety of living organisms using starch as carbon and energy source and also one of the most abundant products of the enzyme industry used mainly for the industrial production of starch derivatives. In spite of the extensive studies concerning the structure and properties of α-amylase [1,2] and the numerous reports in the literature concerning transferase reactions with glucosidases particularly in the presence of alcohols [3–9], little attention has been paid to alcoholysis with α-amylases.

In general, glucosidases may be classified as 'retaining' and 'inverting' enzymes based on the anomeric configuration of the product, that can either retain or invert the anomeric configuration of the substrate. α-Amylases are enzymes acting through a 'retention mechanism' that can also act as transferases, catalyzing the synthesis of oligosaccharides using sugars instead of water as acceptors of the transferring oligosaccharides [10,11]. Additionally, amylases have been classified as 'liquefying' or 'saccharifying' according to their degree of starch depolymerization (saccharifying amylases achieving a higher degree of hydrolysis) [2]. Small molecular weight alcohols may also act as acceptors in the alcoholysis reactions. Among the retaining glucosidases, various enzymes have been successfully used in the presence of alcohols. This is the case with β-galactosidase [3,4], β-xylosidase [5], β-fructofuranosidase [6,7] and β-glucosidase [8,9], which have been proposed for the synthesis of alkyl-glucosides, used in the industry due to their tenso-active properties. Considering the abundance of starch as raw material and substrate, there is an enormous potential in the use of α-amylase for this type of reactions.

Matsubara [12] has shown that α-amylase from *Aspergillus oryzae* (taka-amylase) is able to carry out alcoholysis reactions transferring maltose to methanol, ethanol and butanol when phenyl- and *p*-nitrophenyl-α-maltoside are used as substrates. Taka-amylase is also capable of transferring glucose units from low polymerization degree oligosaccharides to polyols [13]. On the other hand, Nishimura et al. [14] purified a saccharifying α-amylase from *Bacillus subtilis* X-23, capable of transferring glucose and even oligosaccharides to hydroquinone when maltotriose, maltopentose and soluble starch are used as substrates. However, the information in the literature concerning the behavior of the amylases in alcoholysis reactions, particularly in the presence of starch or starch derivatives and alcohols, is scarce.

In this work, the behavior of α-amylases in alcoholysis reactions is described. In particular, the properties of α-amylase from *Aspergillus niger* are studied, using starch and other substrates, with methanol as nucleophilic substitute of water.

2. Materials and methods

2.1. Enzyme properties

α-Amylase was obtained from various commercial sources. The fungal α-amylases produced from *A. niger* were kindly provided by ENMEX (México, D.F.) and 'Proveedor Internacional de Químicos' (México, D.F.) and *A. oryzae* was from Sigma. Bacterial α-amylase from *Bacillus licheniformis* (Thermamyl 120) was purchased from NOVO (México, D.F.) and α-amylase from *Bacillus stearothermophilus* was produced in the laboratory expressing the gene in a recombinant *Escherichia coli* strain, purifying the enzyme as described by Vihinen et al. [15]. Soluble starch and maltodextrins (G4–G10) were purchased from Sigma (USA).

Enzyme activity (hydrolysis) was determined following the initial rate of reducing sugars from 1.2% w/v of starch in 75 mM acetate buffer using the 2,4-dinitro-salicilic reagent [16] with glucose as standard, at pH 4.0 for the fungal and pH 6.0 for the bacterial amylases, both at 60°C, in a total volume of 10 ml. The reaction was started by addition of the enzyme and monitored every 2 min during the first 10 min of the reaction. The activity was obtained from a plot of glucose equivalents produced with the reaction time. One activity unit is defined as the amount of enzyme producing 1 μmol of equivalent glucose per min. As described later, the amount of α-amylase

*Corresponding author. Fax: (52) (73) 172388.
E-mail: agustin@ibt.unam.mx

PII: S0014-5793(99)00667-5

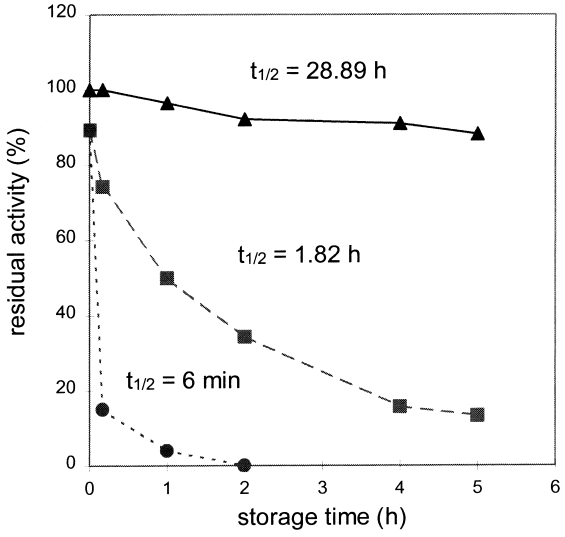

Fig. 1. Stability of *A. niger* α-amylase in the presence of methanol: (▲) 0%, (■) 20% and (●) 40%. The enzyme was incubated at 60°C and pH 4.0.

used in a particular reaction was adjusted in order to have always the same activity in the reaction.

The alcoholysis products were qualitatively analyzed by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). For TLC, 10 cm×10 cm×200 μm Whatman Silica gel 60 plates were used, with butanol, ethanol and water (3:5:2) as solvent. The plates were developed by spraying α-naftol and sulfuric acid in ethanol and heating at 100°C [17] for 5 min. HPLC analysis of the products was carried out in a Water-Millipore chromatography equipment with a refractive index detector and a Nova-Pak aminated (4.6 mm×250 mm) column using acetonitrile and H_2O (70:30) as eluent at a flow rate of 1.4 ml/min. Maltodextrins and methyl-glucoside (Sigma, USA) were used as standards.

2.2. Alcoholysis reactions

Starch solutions were treated at 121°C and 15 psi, for 15 min before the reaction. Afterwards, methanol was added followed by the enzyme to start the reaction (4.8 U/ml of the fungal amylases or 5.4 U/ml of the bacterial enzymes) in a final volume of 10 ml at 60°C. The reaction was followed during 24 h taking 500 μl samples every h and quenching the reaction by boiling for 10 min.

The direct effect of methanol on the enzyme was evaluated by measuring the activity in the presence of 20–40% v/v, while the effect on stability, after incubation at 60°C and pH 4.0 at different times, was followed by dilution and an activity assay. The effect of the starch and methanol concentration on the alcoholysis yield was studied for the fungal amylase at 50, 60 and 70°C and pH 3.0, 4.0 and 5.0. The concentration of starch was varied from 1.2 to 15% w/v with 20% v/v methanol, while the concentration of methanol was varied from 20 to 50% v/v with 3% w/v starch (10 U/ml of enzyme). The alcoholysis products were detected by TLC and HPLC with α-methyl-glucoside (Sigma) as standard. All reactions were conducted for a maximum of 5 h. Relative conversions were determined, taking as 100% the amount of reducing sugars obtained in the absence of methanol after 5 h of reaction at pH 4 and 60°C.

Considering that the alcoholysis products, as observed by HPLC, are a series of methyl-glucosides, mainly from MG1 to MG6 for which no standards are available, in order to quantify alcoholysis, 40 U/ml of glucoamylase (Boehringer, Lakeside, USA) was added to the α-amylase reaction products followed by incubation for 2 h at 40°C. Under these conditions, all alcoholysis and hydrolysis products from α-amylase are further hydrolyzed by glucoamylase to yield methyl-glucoside and glucose. All methyl-glucoside (MG1) is derived from alcoholysis reaction products and was quantified through the linear relation obtained between the MG1 mass, in the range of 0–0.2 mg, and the integrated area from the refractive index detector signal. In the linear regression analysis, a correlation factor of $r^2 = 0.99$ was obtained. Another way used to characterize alcoholysis was to divide the integrated area obtained from the signal of all the alcoholysis products (from MG1 to MG6) by the total integrated area from the signal of both oligosaccharides (G1–G6) and methyl-glucosides (MG1–MG6).

3. Results and discussion

The ability of four α-amylases to carry out alcoholysis was studied. Amylase from *A. niger*, a saccharifying enzyme which produces maltose, maltotriose and some glucose, is capable of alcoholysis as shown by additional products observed when the same reaction is carried out in the presence of methanol (see later). Although less efficient, the same is true for the α-amylase from *A. oryzae*. However, this is not the case for bacterial amylases from *B. licheniformis* and *B. stearothermophilus*, where the same products (maltose, maltotriose and maltopentaose or maltohexaose) are observed in the presence and absence of methanol (results not shown). Similar results have been reported in the literature with other α-amylases and substrates. The saccharifying amylase from *A. oryzae* is able to transfer maltose to methanol and ethanol among other alcohols using aryl-maltoside as substrate [12], while the liquefying amylase from *B. licheniformis* does not transfer glucose to ethanol although the product profile obtained from starch is modified [18]. We have previously shown that transfer reactions in α-amylases produce a higher degree of depolymerization in starch [19] and since transglucosidation and alcoholysis are related [10], we decided to study in more detail the factors affecting the yield of alcoholysis reactions.

3.1. The effect of starch and methanol concentration on alcoholysis

In order to determine the effect of the methanol concentration in alcoholysis reactions, the effect of methanol on the enzyme stability was first studied. In the presence of 40%

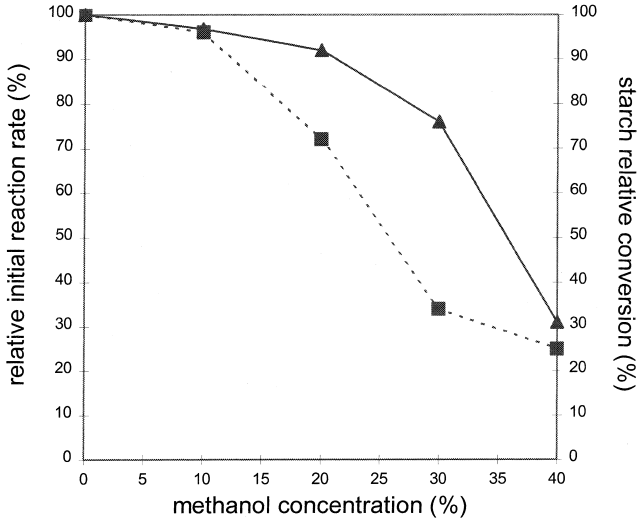

Fig. 2. Effect of the methanol concentration on the initial rate of hydrolysis (■) and the relative substrate conversion (▲). Initial rates are measured at 1.2% w/v starch and 4.8 U/ml, reducing sugars are measured after 5 h of reaction with 15% w/v starch, in both cases at pH 4.0, 60°C of α-amylase from *A. niger*. 100% conversion is taken as the total reducing sugars obtained in the absence of methanol.

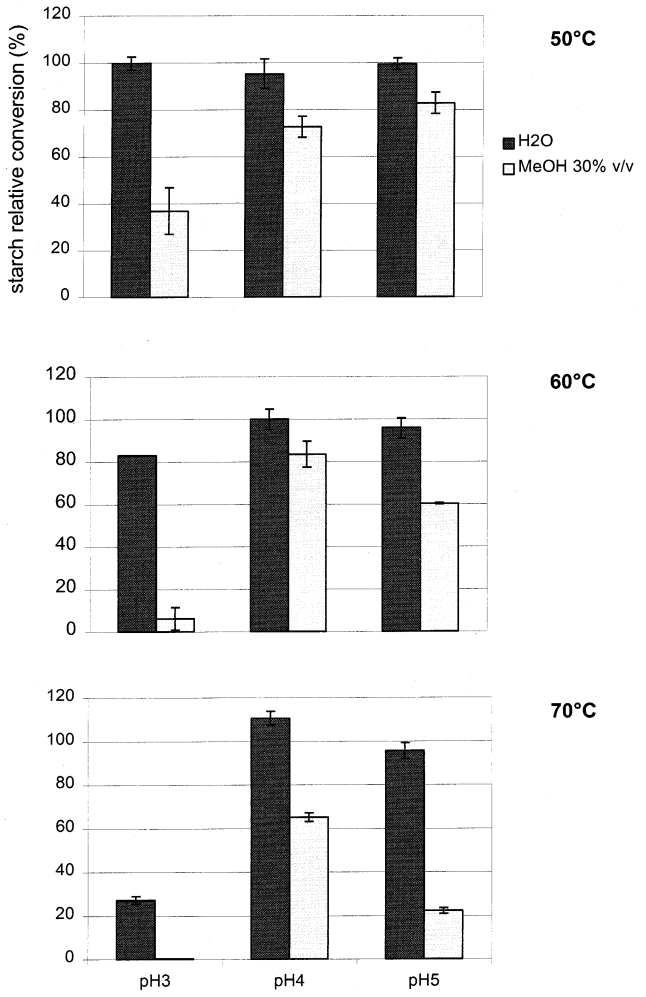

Fig. 3. Effect of the pH on the relative starch conversion by *A. niger* α-amylase at different temperatures in the presence and absence of methanol. Reaction conditions were 15% w/v starch and 30% v/v methanol. A: 50°C, B: 60°C and C: 70°C. Reducing sugars were measured after 5 h of reaction (100% is the total reducing sugars value obtained at pH 4, 60°C, the usual hydrolysis reaction conditions).

methanol, α-amylase rapidly looses activity, with a half-life of 6 min at 60°C (Fig. 1). At 20% methanol, the drop in activity can be described by first order kinetics with a half-life of 1.82 h. This behavior is similar to the one observed for other glucosidases in the presence of methanol or ethanol and limits the range of the alcohol concentration that can be used in the reaction [20,21]. However, there is a stabilizing effect in 15% (w/v) starch, where, for instance, relative conversions higher than 90% are obtained in methanol concentrations up to 20% (v/v) in spite of the low half-life of the enzyme and the significant reduction in the initial rate (Fig. 2). In all cases, the alcoholysis products are the same. At 30% methanol, where the protective effect of starch is evident, the effect of the pH and temperature on alcoholysis was studied. Although there is always some degree of inhibition by methanol, the best reaction conditions for alcoholysis are pH 4 and 60°C, similar to those of hydrolysis (Figs. 3 and 4). This is not surprising since even in non-aqueous solution, pH reaction conditions are similar to those in aqueous medium [22]. It has also been previously observed, in porcine pancreatic α-amylase, that starch can stabilize the enzyme, binding at the boundaries of the three domains present in all α-amylases [23].

Additionally, it has been shown that increases in the substrate concentration favor transglucosilation with a fungal α-amylase [13]. As alcoholysis is a particular case of a transferase reaction, we explored the effect of the starch concentration on the alcoholysis activity, using 20% (v/v) of methanol. Increasing the starch concentration results in the increase of methyl-α-glucoside, the product with an *Rf* of 0.74, and methyl-α-maltoside, as observed by TLC (Fig. 4I). This may be explained in terms of the low water activity in high concentrations of starch. A similar experiment was conducted increasing the methanol concentration while maintain-

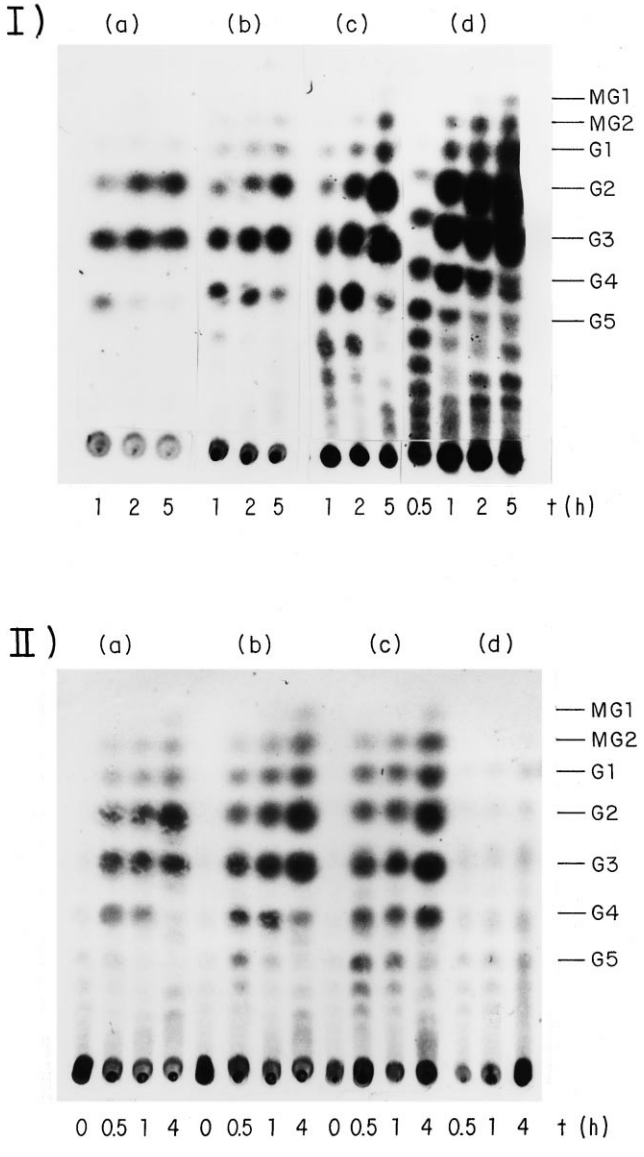

Fig. 4. TLC of alcoholysis reaction products at different reaction times. (I) Effect of the starch concentration on alcoholysis: (a) 1.25%, (b) 2.5%, (c) 6% and (d) 15% w/v of starch, in the presence of 20% v/v methanol. (II) Effect of the methanol concentration on alcoholysis: (a) 20% (b) 30%, (c) 40% and (d) 50% v/v of methanol with 3% w/v of starch. In all cases, using 10 U/ml α-amylase from *A. niger* at pH 4.0 and 60°C. MG1 = methyl-glucoside, MG2 = methyl-maltoside, G1 = glucose, G2 = maltose, G3 = maltotriose, G4 = maltotetraose, G5 = maltopentaose.

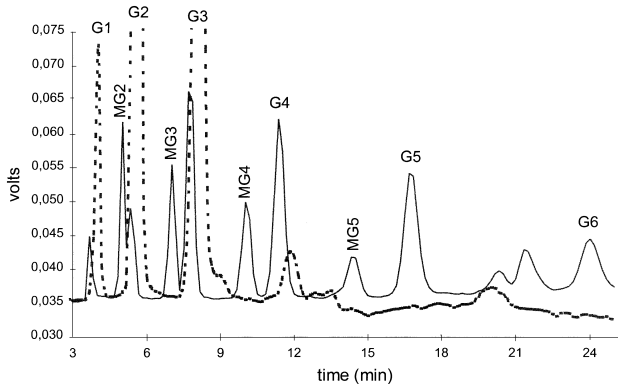

Fig. 5. HPLC chromatogram of the reaction products with α-amylase from *A. niger* at pH 4.0, 60°C with 6% w/v of starch. The dotted line (·····) represents the reaction products in water, while the continuous line (———) represents the products obtained in the presence of 40% v/v methanol. MG2 = methyl-maltoside, MG3 = methyl-maltotrioside, MG4 = methyl-maltotetraoside, MG5 = methyl-maltopentaoside, G1 = glucose, G2 = maltose, G3 = maltotriose, G4 = maltotetraose, G5 = maltopentaose, G6 = maltohexaose.

ing 3% w/v starch (Fig. 4II). The TLC plates show a higher concentration of high molecular weight products, due to the loss of enzyme activity with methanol. Nevertheless and as expected, there is a higher proportion of alcoholysis products. We conclude that a concentration as high as 40% (v/v) methanol may be employed for alcoholysis, provided that a high concentration of starch is also used (e.g. 6% w/v).

3.2. Alcoholysis products

Each time that a starch molecule reaches the active site of an α-amylase molecule and an intermediate is formed, the leaving group may be transferred to water or to methanol. The selectivity of this transfer depends on the concentration of nucleophiles and their specificities defined by their corresponding rate constants. The products of a reaction carried out with 6% (w/v) starch in the presence of 40% (v/v) methanol were directly compared with the hydrolysis products obtained at the same conditions without methanol. For each oligosaccharide obtained, there is also the corresponding alkylated product (Fig. 5). This is an interesting feature of α-amylase, particularly suited for the synthesis of alkyl-glucosides with a controlled number of glucose molecules in the hydrophilic side of the chain. A second observation concerns the specificity of the reaction. As in almost all products, the oligosaccharides are produced in a higher concentration than the corresponding alkylated product. This is in part the consequence of the high water concentration in the reaction medium. However, there is more methyl-maltoside than maltose. As this is an α-amylase producing mainly maltose after extensive hydrolysis of starch (as evident from the products shown in the absence of methanol), it is striking that also the main product transferred to methanol is maltose. The specificity is in part explained by the asymmetric distribution of the catalytic site in α-amylase, which consists of seven subsites, with the catalytic site located between sites 5 and 6 [24]. It would be reasonable to expect that a productive intermediate requires some significant occupancy of the subsite. This is achieved with maltose at the donor site, combined, in the case of alcoholysis, with the higher accessibility of a small acceptor and competing nucleophile such as methanol.

A comparison of the alcoholysis and hydrolysis reactions was carried out from the HPLC chromatograms. In a reaction with 6% w/v starch and 40% v/v methanol, 29.7% of the total area of the products corresponds to alcoholysis products. Alternatively, an indirect quantitative measurement of alcoholysis was adapted from the method developed by Nishimura et al. [14] for the analysis of oligoglucosides obtained in the presence of hydroquinone. The method consists on the addition of glucoamylase free of α-amylase activity (to avoid additional alcoholysis) to the α-amylase alcoholysis reaction products. Glucoamylase is an exo-amylase that acts through an inverting mechanism from the non-reducing end of oligosaccharides producing glucose. In our system, the enzyme transforms all products to glucose and methyl-glucoside.

Methyl-glucoside produced after glucoamylase treatment under different alcoholysis reaction conditions was measured by HPLC (Table 1). More alcoholysis is obtained with α-amylase from *A. niger* than with the enzyme from *A. oryzae*. In a similar way, more alcoholysis products are obtained at a high substrate concentration, as demonstrated by the higher amounts of methyl-glucoside obtained with 15% (w/v) starch, actually, 3.8 times the amount obtained at 6% (w/v). When various substrates are compared at the same concentration (6% w/v), starch seems more efficient than lower molecular weight substrates in terms of transfer to methanol. This is also related to the low water content at high starch concentrations, favoring transglucosidase activity and enzyme stability, as already mentioned above. A similar result was reported by Nishimura et al. [14] when glucosilating hydroquinone with a purified α-amylase, obtaining a higher efficiency with starch compared to maltotriose and maltopentaose.

4. Conclusions

The potential of the saccharifying α-amylase from *A. niger* for the synthesis of methyl-glucosides from starch was demonstrated. Reaction conditions involving a high concentration of starch result in high alcoholysis conversions and although the enzyme is affected by methanol, in the presence of starch, methanol concentrations as high as 40% v/v may be used. The major end product obtained in water (maltose) corresponds to the major alcoholysis end product (methyl-maltoside), which is obtained in higher concentrations than maltose. We propose that this is due to the particular features of the active site in *A. niger* α-amylase leading to a preferred product transfer to small alcohols. If this is correct, then, other product-specific α-amylases, such as those produced by *Pseudomonas stutzeri* or *Aerobacter aerogenes* producing maltotetraose or mal-

Table 1
Efficiency of the alcoholysis reaction of α-amylase from *A. niger* as measured by the total methyl-glucoside produced after digestion with glucoamylase (initial methanol concentration 40% v/v)

Substrate	[S] (% w/v)	Methyl-glucoside (mg/ml)
Starch	6	2.32
Starch[a]	6	0.76
Hydrolized starch[b]	6	0.90
Maltodextrins[c]	6	1.19
Starch	15	8.90

[a]α-Amylase from *A. oryzae*.
[b]Addition of α-amylase 10 min before methanol.
[c]Maltodextrins from Sigma, oligosaccharides G4–G10.

tohexaose [2], would also preferentially produce the corresponding alkylated products.

We have already demonstrated that the combined action of hydrolytic and transferase activities results in a more extensive degradation of starch, combining the transferase activity of cyclodextrin glucosyl transferase and the hydrolytic activity of α-amylase from *B. licheniformis* [19]. This seems to be the case with saccharifying amylases where both activities are involved in a more intensive degradation of starch. The implication of this conclusion is important from both the basic and applied point of views, as not only a more efficient and specific production of starch degradation products may be obtained through a particular combination of activities, but must of all, as the understanding of the structural features responsible for hydrolytic and transferase activities in amylases may lead to the construction of more efficient enzymes.

Acknowledgements: The authors thank the technical support of F. González. This project was supported by DGAPA/UNAM project number 212696.

References

[1] Wong, D.W.S. (1995) in: Food Enzymes, Structure and Mechanism, pp. 37–84, Chapman and Hall, New York.
[2] Fogarty, W.M. (1983) in: Microbial Enzymes and Biotechnology (Fogarty, W.M., Ed.), pp. 1–92, Applied Science Publishers LTD, New York.
[3] Stevenson, D.E., Stanley, R.A. and Furneaux, R.H. (1993) Biotechnol. Bioeng. 42, 657–666.
[4] Moreno, A., Salgado, L., Vazquez-Duhalt, R. and López-Munguía, A. (1998) J. Mol. Catal. B: Enzym., p. 206 (in press).
[5] Shinoyama, H., Kamiyama, Y. and Yasui, T. (1988) Agric. Biol. Chem. 52, 2197–2202.
[6] Straathof, A.J.J., Vrijenhoef, J.P., Sprangers, E.P.A.T., van Bekkum, H. and Kieboom, A.P.G. (1998) J. Carbohydr. Chem. 7, 223–238.
[7] Rodríguez, M., Gómez, A., González, F., Bárzana, E. and López-Munguía, A. (1996) Appl. Biochem. Biotechnol. 59, 167–175.
[8] Chahid, Z., Montet, D., Pina, M. and Graille, J. (1992) Biotechnol. Lett. 14, 281–284.
[9] Vulfson, E.N., Patel, R., Beecher, J.E., Andrews, A.T. and Law, B.A. (1990) Enzym. Microb. Technol. 12, 950–954.
[10] Sinnott, M.L. (1990) Chem. Rev. 90, 1171–1202.
[11] Allen, J.D. and Thoma, J.A. (1978) Biochemistry 17, 2338–2344.
[12] Matsubara, S. (1961) J. Biochem. 49, 226–231.
[13] Graber, M. and Combes, D. (1990) Biotechnol. Bioeng. 36, 12–18.
[14] Nishimura, T., Kometani, T., Takii, H., Terada, Y. and Okada, S. (1994) J. Ferment. Bioeng. 78, 31–36.
[15] Vihinen, M., Ollikka, P., Niskanen, J., Meyer, P., Suominen, H., Karp, M., Holm, L., Knowles, J. and Mäntsälä, P. (1990) J. Biochem. 107, 267–272.
[16] Miller, G.L. (1959) Anal. Chem. 31, 426–428.
[17] Fried, B. and Shelma, J. (1986) In: Thin Layer Chromatography Techniques and Applications, 2nd edn., Chap. 17, p. 287, Marcel Dekker, New York and Basel.
[18] Blakeney, A.B. and Stone, B.A. (1985) FEBS Lett. 186, 229–232.
[19] Del Río, G., Morett, E. and Soberon, X. (1997) FEBS Lett. 416, 221–224.
[20] Ulbrich-Hofmann, R. and Selisko, B. (1993) Enzym. Microb. Technol. 15, 33–41.
[21] Rodríguez, M., Gómez, A., González, F., Bárzana, E. and López-Munguía, A. (1997) J. Mol. Catal. B 2, 299–306.
[22] Fitzpatrick, P.A., Steinmetz, A.C., Ringe, C. and Klibanov, A. (1993) PNAS USA Sep 15 90(18), 8653–8657.
[23] Qian, M., Haser, R. and Payan, F. (1995) Protein Sci. 4, 747–755.
[24] Matsuura, Y., Kusunoki, M., Harada, W. and Kakudo, M. (1984) J. Biochem. 95, 697.