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RESEARCH ARTICLE

Modification of the activity of an α-amylase from *Bacillus licheniformis* by several surfactants

Vicente Bravo Rodríguez*
Departamento de Ingeniería Química
Universidad de Granada
Avd. Fuentenueva s/n
18071 Granada, Spain
Tel: 34 958 243310
Fax: 34 958 248992
E-mail: vbravo@ugr.es

Encarnación Jurado Alameda
Departamento de Ingeniería Química
Universidad de Granada
Avd. Fuentenueva s/n
18071 Granada, Spain
Tel: 34 958 243307
Fax: 34 958 248992
E-mail: ejurado@ugr.es

Juan Francisco Martínez Gallegos
Departamento de Ingeniería Química
Universidad de Granada
Avd. Fuentenueva s/n
18071 Granada, Spain
Tel: 34 958 243314
Fax: 34 958 248992
E-mail: jfmart@ugr.es

Antonia Reyes Requena
Departamento de Ingeniería Química
Universidad de Granada
Avd. Fuentenueva s/n
18071 Granada, Spain
Tel: 34 958 249018
Fax: 34 958 248992
E-mail: areyesr@ugr.es

Ana Isabel García López
Departamento de Ingeniería Química
Universidad de Granada
Avd. Fuentenueva s/n
18071 Granada, Spain
Tel: 34 958 249018
Fax: 34 958 248992
E-mail: vbravo@ugr.es

Joaquim Manuel Sampaio Cabral
Centro de Engenharia Biológica e Química
Instituto Superior Técnico
Universidade Técnica de Lisboa
Av. Rovisco Pais
1049-001 Lisboa, Portugal
Tel: 351 218 419 063
Fax: 351 218 419 062
E-mail: joaquim.cabral@ist.utl.pt

Pedro Fernandes
Centro de Engenharia Biológica e Química
Instituto Superior Técnico
Universidade Técnica de Lisboa
Av. Rovisco Pais
1049-001 Lisboa, Portugal

Tel: 351 218 419 189
Fax: 351 218 419 062
E-mail: pedro.fernandes@mail.ist.utl.pt

Luis Joaquim Pina da Fonseca
Centro de Engenharia Biológica e Química
Instituto Superior Técnico
Universidade Técnica de Lisboa
Av. Rovisco Pais
1049-001 Lisboa, Portugal
Tel: 351 218 419 139
Fax: 351 218 419 062
E-mail: lfonseca@alfa.ist.utl.pt

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Keywords: α-amylase, alkyl polyglycosides, enzymatic activity, fatty alcohol ethoxylates, linear alkyl benzene sulfonate, nonyl phenol ethoxylate.

Abbreviations: AE: enzymatic activity without surfactant
AE_T: enzymatic activity in presence of surfactant
AER: relative enzymatic activity
CMC: critical micelle concentration
HLB: Hydrophilic Lipophilic Balance
KNU: Kilo Novo Unit
LAS: linear alkyl benzene sulfonate
NU: Novo Unit
SDS: sodium dodecyl sulphate

The influence of different commercial surfactants on the enzymatic activity of a commercial α-amylase from *Bacillus licheniformis* (Termamyl 300 L) has been studied. As non-ionic surfactants, alkyl polyglycosides (Glucopon® 215, Glucopon® 600 and Glucopon® 650) were studied, as were fatty alcohol ethoxylates (Findet 1214N/23 and Findet 10/15), and nonyl phenol ethoxylate (Findet 9Q/21.5NF). Also, an anionic surfactant, linear alkyl benzene sulfonate (LAS) was assayed. In general, none of the non-ionic surfactants studied, except Findet 10/15, vary substantially the enzymatic activity. Findet 10/15 has the strongest hydrophobic character and reduces the enzymatic activity more significantly the greater its concentration. Regarding LAS, this surfactant significantly depressed enzymatic activity, presumably due to the electrostatic interactions caused by its anionic character.

Nowadays, amylases (α-amylases, β-amylases and glucoamylases) represent one of the most important enzyme groups within the field of biotechnology. These enzymes are present in numerous biotechnological and industrial applications, including their use in detergent formulations (Tanaka and Hoshino, 1999). The use of enzymes in detergents offers multiple advantages from an environmental standpoint: energy savings on using lower washing temperatures, replacement or reduction of other more environmentally harmful components, lack of negative effects on the sewage-water-treatment systems, and absence of risk for aquatic wildlife.

One of the main problems affecting the action of these enzymes in detergent formulations is the possible influence of surfactants on enzymatic hydrolysis, due to possible interactions of the surfactant both with the reaction substrate as well as with the enzyme.

With respect to the substrate, in the case of starch, it is known that amylose, mainly, and to a lesser extent amylopectin can interact with surfactants, giving rise to inclusion complexes (Svensson et al. 1996; Lundqvist et al. 2002a; Lundqvist et al. 2002b; Lundqvist et al. 2002c; Tanaka and Hoshino, 2002; Hoshino and Tanaka, 2003). The formation of these complexes between the surfactants and starch can hamper their enzymatic hydrolysis with amylases; thus, it has been confirmed that the formation of surfactant complexes with amylose hinders enzymatic hydrolysis with β-amylase, being only partially hydrolysable (Kim and Robinson, 1979).

With regard to the enzyme, many surfactants that interact with proteins can present in their structure different electrical charges and different hydrophobic and hydrophilic groups which alter the secondary and tertiary structures of proteins. In particular, many enzymes are unstable in solutions of anionic surfactants such as sodium dodecyl sulphate (SDS), sodium dodecyl benzene sulfonate and sodium ether sulphate (Tanaka and Hoshino, 1999; Tanaka and Hoshino, 2002). In addition, the formation of micelles can also affect enzymatic kinetics. It has been demonstrated that the hydrolysis rates of amylose by α-amylase from *Bacillus amyloliquefaciens* and *Bacillus licheniformis* vary in the presence of SDS; the reaction rate

* Corresponding authors

increases at concentrations lower than the critical micelle concentration of the surfactant and decreases over this level, this effect being less pronounced on the enzyme from *Bacillus licheniformis* than on the enzyme from *Bacillus amyloliquefaciens* (Tanaka and Hoshino, 2002).

Table 1. Critical micelle concentration, hydrophilic-lipophilic balance, and moisture content of the different commercial surfactants assayed.

Surfactant	CMC (g/L)	HLB(1)	H (%)
Glucopon 215	0.241	13	37.0(1)
Glucopon 600	0.028	11.2	46.6(1)
Glucopon 650	0.073	11.9	50.4(1)
Findet 10/15	0.152	9.6	0.423(1)
Findet 1214N/23	0.021	14.4	0.309(1)
Findet 9Q/21.5NF	0.034	12.8	1.4
LAS	1.018	-	0(2)

(1) Data obtained from the results of Bravo Rodríguez et al. (2005).
(2) Data provided by the manufacturer.

In addition, some enzymes are more stable in the presence of non-ionic surfactants than in the presence of anionic surfactants. The formation of micelles of anionic surfactants can alter the conformation of the protein due to the existence of strong electrostatic and hydrophobic interactions between the micelles and the proteins (Montserret et al. 2000; Hagihara et al. 2002). On the contrary, non-ionic surfactants do not alter the conformation of the protein, since they do not cause any electrostatic interaction (Russell and Britton, 2002) and, moreover, some non-ionic surfactants increase the catalytic activity of enzymes. On studying the effect of the non-ionic surfactant polyoxyethylene mono-N-dodecyl ether (Brij 35) on the enzymatic hydrolysis of amylopectin from potato with an α-amylase from *Bacillus amyloliquefaciens*, it was found that the reaction rate raised with an increasing concentration of the surfactant above its critical micelle concentration while the non-aggregated surfactant molecules did not boost the reaction rate (Hoshino and Tanaka, 2003). It has been shown that amylopectin binds to surfactant and that enzyme binds to the micelles of the surfactant in such a way that the catalysis appears to be more efficient in the micellar pseudophase than in the aqueous pseudophase. This most likely results from the high enzyme and amylopectin concentrations in the micellar pseudophase. Also, it has been verified that fatty alcohol ethoxylates stabilize proteases in the presence of anionic surfactants such as LAS, thereby preventing the loss of the enzymatic activity (Russell and Britton, 2002). Finally, it should be highlighted that the alkyl polyglycosides are capable of increasing enzyme stability (proteases, lipases, amylases, and cellulases) in liquid-detergent formulations, impeding the loss of activity during detergents storage (Von Rybinski and Hill, 1998).

When these facts are taken into account, it becomes evident that in order to effectively use α-amylases in detergent formulations it is necessary to study the effect that different surfactants can cause in its enzymatic activity. In the present work, the influence of several non-ionic surfactants, alkyl polyglycosides, fatty alcohol ethoxylates, and nonyl phenol ethoxylate is studied in relation to the activity of a commercial α-amylase, comparing them with a classical anionic surfactant, linear alkyl benzene sulfonate (LAS).

MATERIALS AND METHODS

An α-amylase of bacterial origin (*Bacillus licheniformis)* from Novozymes A/S, Termamyl® 300 L Type DX was used. This enzyme is used specifically in detergent formulations for laundry and dishwashers. It presents an activity, according to the manufacturer, of 300 KNU/g, where 1 KNU (Kilo Novo Unit) is defined as the amount of enzyme which hydrolyses 4870 mg (on a dry basis) of soluble Merck starch (Erg. B 6, lot number 6380528) per hour under standard conditions, pH 5.6, 37ºC, and Ca^{2+} concentration 0.0003 M (Novozymes A/S, 2001a).

As non-ionic surfactants, alkyl polyglycosides Glucopon® 215 CS UP, Glucopon® 600 CS UP, and Glucopon® 650 EC (from Cognis Deutschland GmbH&Co) were used, as well as fatty alcohol ethoxylates, Findet 10/15 and Findet 1214N/23 (Kao Corporation S.A.), and nonyl phenol (with 9.5 moles of ethylene oxide) under the commercial name of Findet 9Q/21.5NF (Kao Corporation S.A.). In addition, an anionic surfactant was used, LAS (linear alkyl benzene sulfonate), from Kao Corporation S.A.. Table 1 lists the critical micelle concentration values (CMC), hydrophilic-lipophilic balance (HLB), and initial moisture content of the commercial surfactant (H, % w/w), for each surfactant. The CMC values were established by measuring the surface tension of surfactant solutions with different concentrations at 37ºC, using a tensiometer model Tensiometer K11 (KRÜSS GmbH) equipped with a 2 cm platinum plate. The moisture content of Findet 9Q/21.5NF was determined from two dried samples in an infrared balance, model AD-4714A (A&D Co., Ltd.), at 102ºC for 30 min.

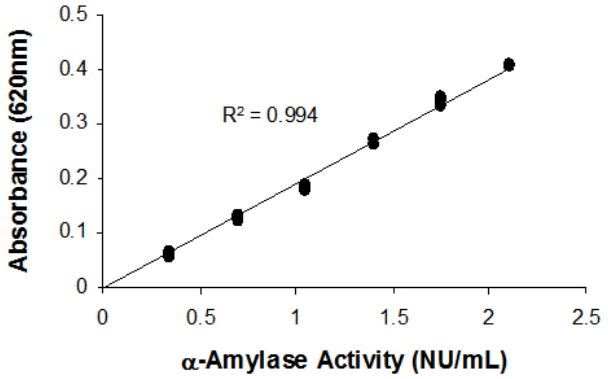

Figure 1. Standard calibration curve for the α-amylase activity with Phadebas® tablets.

To determine the activity of the α-amylase in the presence of the surfactants, a standard method was used (Novozymes A/S, 2001b) based on the kit Phadebas® Amylase Test from

Table 2. Surfactant concentration, enzymatic activity in the presence of surfactant (mean of duplicated experiments), enzymatic activity in the absence of surfactant (mean of duplicated experiments), relative enzymatic activity (mean of duplicated experiments) and corresponding standard deviation, for the different commercial surfactants assayed.

	pH = 7.3		T = 37ºC		
Surfactant	C (g/L)	AE_T (NU/ml)	AE (NU/ml)	AER (%)	STDV (%)
Findet 1214N/23	0.060	1.68	1.78	94.6	0.6
Findet 1214N/23	0.060	1.66	1.78	93.2	1.1
Findet 1214N/23	0.006	1.75	1.79	97.5	0.6
Findet 1214N/23	0.006	1.74	1.78	97.8	0.0
Findet 10/15	1.0	0.70	1.75	40.1	0.5
Findet 10/15	0.2	1.36	1.75	77.5	0.0
Findet 9Q/21.5NF	0.025	1.53	1.67	91.3	2.8
Findet 9Q/21.5NF	0.015	1.51	1.67	89.7	0.3
Glucopon 215	1.2	1.86	1.75	106.2	3.4
Glucopon 215	0.6	1.79	1.75	101.9	0.8
Glucopon 600	0.10	1.89	1.75	108.0	1.6
Glucopon 600	0.05	1.88	1.75	107.5	0.0
Glucopon 650	0.3	1.79	1.75	102.4	5.6
Glucopon 650	0.1	1.79	1.75	102.5	4.6
LAS	0.8	1.06	1.67	63.1	0.2
LAS	0.2	1.04	1.67	62.0	3.7

PHARMACIA Diagnostics AB. This method uses Phadebas® tablets as reaction substrate, which contains an insoluble coloured starch complex that releases soluble blue fragments when hydrolysed by the α-amylase. The colour can be measured spectrophotometrically at 620 nm, the absorbance measured being a function of the enzymatic activity (Ceska et al. 1969). Taking as a reference an enzyme of known activity enables the calculation of the enzymatic activity in the presence or absence of surfactants. For this a standard calibration curve of Termamyl solutions with different enzymatic activity was made (Figure 1), under the experimental conditions 37ºC and pH 7.3.

The relative enzymatic activity, AER, was defined as the quotient between the activity in the presence of the surfactant, AE_T, and the activity in its absence, AE. For each surfactant, the relative activity of the enzyme was measured at two different concentrations. Duplicate runs, at least, were performed in each case.

RESULTS AND DISCUSSION

Table 2 lists the mean enzymatic activities of each duplicated experiment in the presence and absence of the surfactant, as well as the mean value of the resulting relative enzymatic activity for each duplicated assay together with its standard deviation (STDV) for each of the concentrations of the commercial surfactants assayed.

First, four duplicated experiments were performed with Findet 1214N/23, two at concentrations above its CMC and another two at concentrations below its CMC (Table 2). The mean AER is slightly higher at concentrations below the CMC, when matched to the AER at concentrations above the CMC, the reverse situation to that described by other authors, who reported an increase in the reaction rate at concentrations above the CMC for non-ionic surfactants (Hoshino and Tanaka, 2003). Nevertheless, it should be pointed out that the differences between the mean AER values at concentrations above and below the CMC are very small, these values also being very close to 100%; it can therefore be concluded that the fatty alcohol ethoxylate Findet 1214N/23 does not significantly affect the enzymatic activity.

The next surfactant studied was another fatty alcohol ethoxylate Findet 10/15 (Table 2). Given that in the case of Findet 1214N/23 no significant differences were detected between surfactant concentrations above and below the CMC, only experiments at surfactant concentrations higher than its CMC were made. With Findet 10/15, very significant losses in enzymatic activity are noted and this loss in activity increases with the concentration of the surfactant. This situation was not foreseeable, since this is a non-ionic surfactant, which has no electrostatic interactions with the enzyme, and being known the capacity of the fatty alcohol ethoxylates to stabilize proteases in the presence of anionic surfactants (Russell and Britton, 2002). Thus such a sharp decline in the enzymatic activity was unexpected. However, it should be pointed out that Findet 10/15

presents the lowest HLB of all non-ionic surfactants assayed, (Table 1). Also, its HLB is slightly lower than 10, that is, the lipophilic groups predominates over the hydrophilic ones, and thus its greater hydrophobic character may determine a greater interaction with the enzyme and hence a reduction in the enzymatic activity which is more significant as the surfactant concentration increases.

The last non-ionic ethoxylate surfactant studied was Findet 9Q/21.5F. Concentrations close to its CMC, but below this value (surfactants are usually used at concentrations under CMC but near to this value), were assayed showing a very slight loss of enzymatic activity in the presence of surfactant, which furthermore does not depend on the surfactant concentration used (Table 2).

In the case of alkyl polyglycosides, three surfactants were assayed: Glucopon® 215, Glucopon® 600, and Glucopon® 650, the results of which are listed in Table 2. All these surfactants were assayed at concentrations above their corresponding CMC, resulting in a slight increase in the enzymatic activity in all cases, in agreement with the work by Hoshino and Tanaka (2003). On the other hand, these results are the opposite of the observations with the fatty alcohol ethoxylates assayed. In any case, given that the increase in enzymatic activity that is observed in the presence of these alkyl polyglycosides is very small, it can be concluded that the latter do not significantly affect the activity of the α-amylase tested.

Finally, the effect of LAS on the enzyme activity was studied, testing concentrations lower than its CMC (Table 2). It is found that LAS, apart from its now known capacity for destabilizing proteases (Russell and Britton, 2002), is also capable of significantly decreasing the activity of the α-amylase studied, even at concentrations lower than its CMC, as opposed to the results previously published with another anionic surfactant, SDS (Tanaka and Hoshino, 2002). This noteworthy loss in enzymatic activity is most likely due to the electrostatic interactions inherent to the anionic character of LAS.

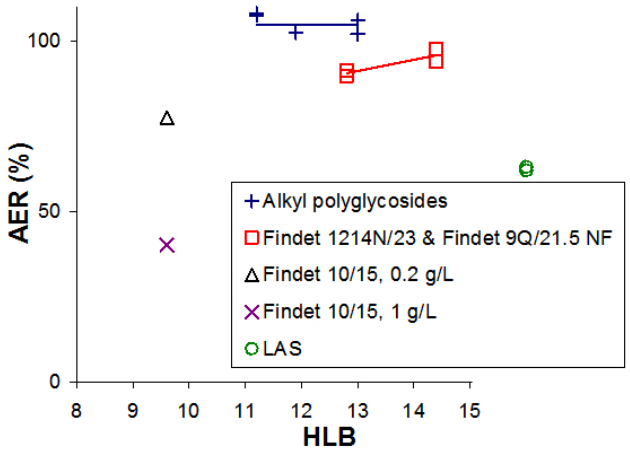

Figure 2. Influence of the HLB on the relative enzymatic Activity.

To conclude, the mean relative enzymatic activity for each surfactant concentration has been represented versus its HLB (Figure 2). In addition, it has been included, outside the HLB scale and for comparative purposes, the values corresponding to LAS. For alkyl polyglycosides, the enzymatic activity remains approximately constant and independent of the HLB value. On the contrary, considering the ethoxylate surfactants, the enzymatic activity declines on decreasing the HLB, in such a way that the less hydrophilic character of the nonyl phenol ethoxylate (Findet 9Q/21.5F) with respect to the Findet 1214N/23 could be the cause of the small loss in enzymatic activity detected in the assays made with Findet 9Q/21.5F (Table 2). Furthermore Brij 35 had been found to increase the hydrolytic rate of *Bacillus amyloliquefaciens* α-amylase up to 65% when the surfactant was added at concentrations over 1.0% (wt/vol), which is higher than its CMC (Hoshino and Tanaka, 2003). Bearing in mind that Brij 35 is a fatty alcohol ethoxylate with 12 atoms of carbon in its alkyl chain and 23 ethylene oxide moles per mol of surfactant, its HLB has also been calculated, obtaining a value 17.2. The HLB value of Brij 35 is higher than any of the ethoxylated surfactants assayed and, in agreement with the results obtained in the present work, it has the highest relative hydrolytic rate. For HLB values lower than 10, in the case of Findet 10/15, where the hydrophobic groups rules out in the molecule considered, there is a very significant loss of enzymatic activity, which in addition increases with the surfactant concentration.

From the results, it can be deduced that when α-amylase from *Bacillus licheniformis* is to be used in detergent formulations, and from the standpoint of the possible alteration of its enzymatic activity, the most suitable surfactants to be included in these formulations would be alkyl polyglycosides, as they even tend to increase the activity somewhat. In the case of the ethoxylate surfactants studied a loss of enzymatic activity is observed, being more significant the lower the surfactant HLB value. This loss is proved to be significant also in the case of the classical anionic surfactant LAS.

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