ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 2 (1997) 299–306

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Stability of invertase in alcoholysis reactions with methanol

M. Rodríguez [a], A. Gómez [a], F. Gónzalez [a], E. Barzana [b], A. López-Munguía [a,*]

[a] *Instituto de Biotecnología, UNAM, Apartado Postal 510-3. Cuernavaca, Mor. 62271, Mexico*
[b] *Facultad de Química, UNAM, C.U. Mexico 04510 D.F., Mexico*

Received 5 November 1996; accepted 2 December 1996

Abstract

The effect of methanol on the stability and structure of invertase is reported. It is shown by intrinsic fluorescence, that methanol induces irreversible changes in structure leading to its deactivation, while concentrations higher than 20% v/v also lead to aggregation and eventual precipitation, a phenomenon which does not involve deactivation. On the other hand, low concentrations of methanol change the kinetic behavior of enzyme as it acts as a mixed type inhibitor.

Keywords: Invertase; β-fructofuranosidase; Alcoholysis; Irreversible denaturation; Intrinsic fluorescence

1. Introduction

The chemical synthesis of alkylglycosides is considered a complex reaction. This is particularly due to the possibility of α- or β-configuration of the glycosidic linkage between glycosyl units and to the similar and relatively low reactivity of the multiple hydroxyl groups of glycosides. The chemical methods used to form glycosidic linkages usually employ toxic, expensive and in some cases, explosive compounds; different protection groups need to be used to promote regioselective reactions in order to achieve good stereoselectivity and yield; the process requires several steps and the separation and purification of the final product is difficult [1].

Enzymes such as carbohydrases are particularly suited for the synthesis of alkylglycosides, as they are capable of transferring sugars not only to water but, also to other sugars and to alcohols acting as nucleophiles. In particular β-fructofuranosidase or invertase (E.C. 3.3.1.26) is capable of transferring fructose to water (hydrolysis) and to alcohols (alcoholysis). The yield of the alkyl-fructoside product depends on several factors such as alcohol and water concentration as well as reaction time, as the fructosides can also be hydrolyzed by the enzyme [2]. Reaction conditions may be therefore optimized for maximum fructoside yield.

Although invertase has a high thermal stability, [3] the major drawback in alkylfructoside synthesis is the low enzyme activity and/or stability in the presence of alcohols [4]. Selisko et al. [2] observed that increasing the concentration of soluble alcohols (methanol, ethanol and propanol) increases the rate of alcoholysis, but

* Corresponding author.

PII S1381-1177(96)00063-X

decreases the overall reaction rate due to inactivation of the enzyme by the alcohol, reporting also that the inactivation is irreversible and that the rate of deactivation is proportional to the length of the alcohol carbon chain. Later, Ulbrich-Hofmann and Selisko [5] carried out stability studies with the free enzyme as well as with the enzyme immobilized by covalent linkage in various supports. They found that the enzyme in both forms loses activity at concentrations higher than 50% (v/v) of methanol, with precipitation of the soluble enzyme. They conclude that immobilization does not favor enzyme stability in the presence of methanol. Furthermore, they observed a higher retention of activity of the soluble enzyme at methanol concentrations higher than 50% (v/v). Straathoff et al. [4] propose that β-fructofuranosidase has two binding sites: one specific for fructose and a rather unspecific aglycon binding site with non-polar character for the aglycon, shown by the fact that the enzyme has a higher affinity for butyl-β-D-fructoside (K_m = 5.4 mM) than for sucrose (K_m = 38 mM). The aliphatic alcohols inhibitory effect would be the result of hydrophobic interactions with the aglycon site. This is not the case of methanol which actually gives the highest yields of fructoside among different alcohols. However, the deactivating effect of methanol complicates the long term application of the enzyme, in particular for the production of methyl fructoside. In a recent report we established conditions for the maximum production of methyl-fructoside in alcoholysis reactions using β-fructofuranosidase with methanol in terms of selectivity [6]. In the present paper we quantify and describe enzyme changes in structure and activity in the presence of methanol.

2. Materials and methods

β-fructofuranosidase from baker's yeast (*Saccharomyces cerevisiae*) was obtained from Gist-Brocades. In some experiments whole *S. cerevisiae* cells where employed. In these cases baker's yeast from Nevada Company, (Toluca Edo, Mexico) were used.

Most of the experiments were carried out with the enzyme from Gist-Brocades. The preparation was analyzed by electrophoresis. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was prepared and run as described by of Laemmli [7] using 0.75 mm thick slabs 10% T, 2.7% C; where T denotes the total weight of monomer (acrylamide plus N,N-methylene bis acrylamide) per 100 ml of solution and C denotes the weight of N,N-methylene bis-acrylamide expressed as percentage of total weight monomer. The gel was stained with Coomassie Blue R-250. As shown in Fig. 1, at all concentrations (even with 50 μg of the preparation) one protein band is dominant.

The overall enzyme activity was measured following the release of reducing sugars in 12 ml assay reactions with 60 g/L sucrose solution in acetate buffer 0.05 M, pH 4.6 at 40°C. Reducing sugars were monitored with the 3,5-dinitrosalicilic acid reagent [8]. Samples were taken during the first 10 min from the reaction and an appropriate dilution of the amount of glucose and fructose was determined from a standard made with equimolecular mixtures of the two sugars. One activity unit (U) is defined as the amount of enzyme transforming 1 μmol of sucrose per min.

The soluble enzyme apparent molecular weight in the presence of methanol was also examined by gel permeation HPLC, on a Bio-Sep-Sec-2000 column, at pH 4.6, using 0.05 M acetate buffer as the mobil phase at a flow rate of 1 ml/min, measured with a Waters UV 486 tunable absorbance detector at 280 nm.

2.1. Invertase derivatives

In some experiments whole cells where used instead of the soluble enzyme. In order to avoid autolysis by methanol the cells were crosslinked according to the following procedure: 8 ml of a 16 g/L cell solution were sonicated and mixed

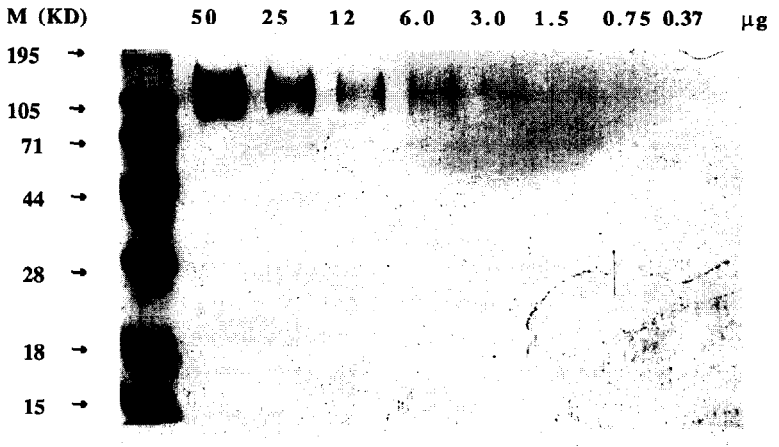

Fig. 1. SDS-polyacrylamide gel electrophoresis of the invertase preparation used for experiment. Samples corresponding to invertase 50 to 0.37 μg and the first lane (M) to molecular mass markers.

with 32 ml of the same intact cell solution. Afterwards, 16 ml of a polyelectrolyte (AS 799, ROHM and HAAS), 4 ml of 0.1 M pH 6 phosphate buffer and 20 ml of a 16% w/v polyetilenimide solution were added. Finally 16 ml of a 4% w/v glutaraldehyde solution were added slowly until aggregation. The precipitated aggregates were washed and kept under refrigeration. A stable aggregate was obtained with 20% activity yield, bearing 24 U/g. Invertase was also immobilized by covalent linkages involving the association of the amino groups of the protein and alumina with glutaraldehyde as crosslinking agent. The aminated alumina was prepared by silanization as described by Weetall [9].

2.2. Effect of methanol on invertase activity

The soluble enzyme (1 mg/ml), the enzyme immobilized in alumina (100 mg/10 ml), whole *S. cerevisae* cells (1.6 mg/ml) or the crosslinked derivative (600 mg/10 ml) were stored at 40°C in acetate buffer 0.05 M pH 4.6 containing methanol (10, 20, 40 and 60% v/v). Samples were taken over a 2 h period and the activity assayed after dilution of the soluble enzyme or after washing the insoluble enzyme preparations with buffer.

2.3. Methanol inhibition

Initial velocity (overall enzyme activity) was measured at various concentrations of sucrose in the presence of different fixed concentrations of methanol, at 40°C and pH 4.6.

2.4. Intrinsic fluorescence

The intrinsic fluorescence emission spectra of invertase was determined in a Perkin Elmer LS50 spectrofluorometer with 15 nm slits excitation and 5 nm slits emission. At an excitation wavelength of 285 nm, emission spectra were recorded between 250 and 500 nm. The spectral center of mass (SCM) was calculated according to the following equation [10]:

$$\lambda_{scm} = \frac{\int I(\lambda)\lambda \, d\lambda}{\int I(\lambda) \, d\lambda}$$

where λ is the wavelength (in nm) and $I(\lambda)$ is the fluorescence intensity at a given wavelength. In all cases, emission fluorescence spectra of samples of the same composition but without the enzyme, were used as control and subtracted from the spectra of experimental samples.

3. Results and discussion

Alcoholysis reactions of invertase with methanol reported in the literature are carried out at various methanol concentrations usually selected on the basis of inhibition of initial reaction rates. When high concentrations of methanol are used (e.g. 50% v/v), sucrose conversion is low [2], due to the additional effect of methanol on enzyme stability. The selection criterium of an appropriate methanol concentration should consider enzyme stability and inhibition, as well as selectivity of the alcoholysis/hydrolysis reaction. Therefore, the stability and inhibition of methanol on different forms of the enzyme were studied.

3.1. Enzyme stability

In Fig. 2 the stability of invertase in the presence of methanol is reported. At alcohol concentrations lower than 20% v/v the effect on stability is negligible during the 4 h of the experiment carried out at 40°C. It is however clear that when the enzyme is in contact with the alcohol solution, the loss of activity takes place in two steps: a rapid drop upon contact of the enzyme with the alcohol, followed by a continuous but slower deactivation rate. It may also be observed that the initial drop is proportional to the alcohol concentration and that concentrations higher than 40% v/v, completely deactivate the enzyme in 30 min. It is also observed that the initial activity drop at concentrations above 20% v/v depends on the way the experiment is conducted, with the lowest drop obtained when the enzyme is added to the alcohol solution already at the final alcohol concentration (results not shown). We have reported 100% conversion of sucrose at 20% v/v [6]. However, from the stability results it may be concluded that concentrations higher than 20% v/v are not suitable for a long term application of the enzyme.

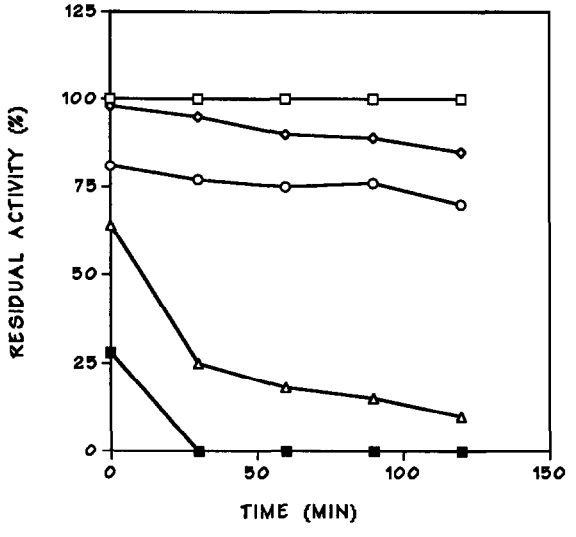

Fig. 2. Effect of methanol concentration on the residual activity of invertase. The enzyme was incubated at 40°C in the presence of various methanol concentrations: (□) control, (◇) 10, (○) 20, (△) 40 and (■) 60% v/v. After dilution, the residual activity was assayed as described in Section 2.

3.2. Enzyme aggregation

Protein aggregation is a well know phenomenon, that may be induced by a change in the dielectric constant of the solution by addition of a solvent such as methanol. In order to study the methanol deactivation effect on invertase, the enzyme was incubated in the presence of 40% v/v methanol. The aggregation state was analyzed in samples after 1 and 2 h of incubation by gel permeation in HPLC. The elution profile is shown in Fig. 3A where it may be observed that during the initial steps, there is an apparent increase in the molecular weight of the soluble enzyme as the retention time of the enzyme in methanol is reduced with time. As was shown in the previous experiments (Fig. 2), after 2 h in 40% v/v methanol, the enzyme is no longer active. However, when the deactivated enzyme is diluted and kept at 25°C it slowly recovers its original molecular weight as can be observed in Fig. 3B. Nevertheless, after dilution we were not able to recover the activity lost in the presence of methanol, showing that aggregation is a reversible phenomenon and is not the cause of inactivation.

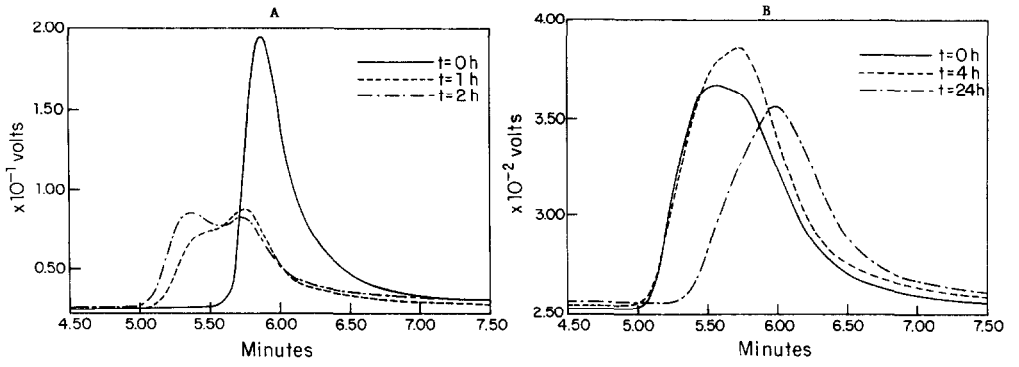

Fig. 3. Effect of methanol on the apparent molecular weight of invertase measured by GPC in HPLC. (A) The enzyme was incubated at pH 4.6, 40°C in 40% methanol and the molecular weight determined at time zero, and after 1 and 2 h. Gel permeation, chromatography profile. (B) After 2 h the sample was diluted (time zero) and incubated. The molecular weight was measured after 4 and 24 h after dilution.

3.3. Conformational modifications

The conformational modifications of invertase in the presence of methanol were studied by intrinsic fluorescence emission. Invertase from *S. cerevisae* has a large number of aromatic aminoacids: 28 tyrosines, 35 phenylalanines and 14 tryptophanes from a total of 517 aminoacids [11]. Samples of the soluble enzyme were incubated in the presence of 20% and 40% v/v methanol for 2 h. Then, the spectrum was measured and the enzyme solution was incubated again at 25°C. Structural changes during incubation were followed by fluorescence. The results are shown in Fig. 4A and B. The fluorescence spectrum of the enzyme in solution is also shown (control). Firstly, there is a clear difference between the control and the enzyme after 2 h in methanol (time zero); at 40% v/v methanol, the loss of activity after 2 h is more than 75% while at 20% v/v it is not higher than 25%. The loss of activity seems to be associated to a fluorescence emission spectrum with a higher intensity at 340 nm. After dilution and incubation, the original spectrum is not recovered and there is a new form of the protein with a higher intensity at around 320 nm which may be associated with disaggregation of the protein.

To complement these observations, the enzyme was incubated in the presence of 10, 20, 30 and 40% v/v methanol at 40°C. At time zero, after immediate addition of the alcohol,

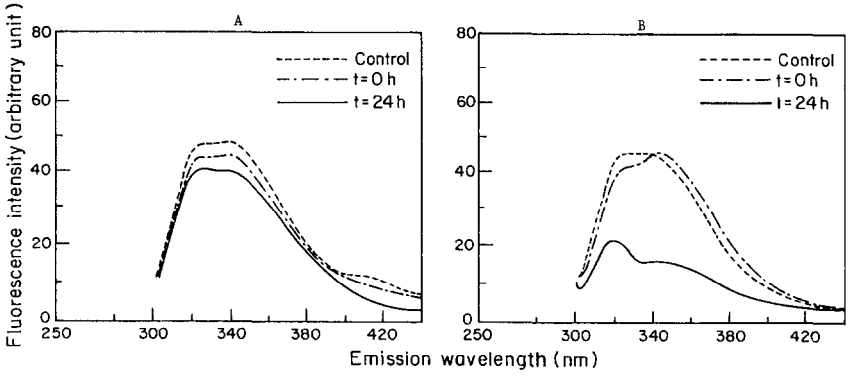

Fig. 4. Intrinsic fluorescence emission spectra of invertase in 20 (A) and 40% v/v (B) methanol. The spectra were measured: in buffer (control), after 2 h of incubation in the corresponding methanol concentration followed by dilution (time zero) and after 24 h incubation.

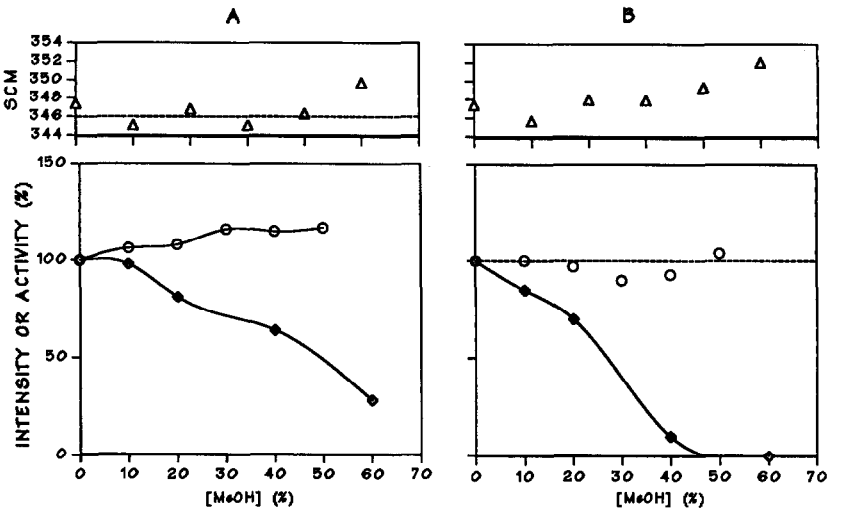

Fig. 5. Intensity of emission fluorescence (○), spectral center of mass (△) and activity (◇) of invertase incubated at pH 4.6 and 40°C at different concentrations of methanol. (A) Immediately after addition of methanol. (B) After 2 h incubation. The results of intensity of fluorescence emission and activity are plotted as percentage of the maximal intensity fluorescence at the spectral center mass.

the spectral center of mass (SCM) from fluorescence spectra of all samples was determined, resulting in a constant value at around 346 nm, while a slight increase in intensity proportional to methanol concentration was observed (Fig. 5A). The SCM was determined again after 2 h of incubation with methanol (Fig. 5B). This time, a slight constant increase in the SCM with increasing methanol concentration, was observed while the intensity seems to remain constant. In both figures the enzyme activity is also reported.

As already shown in Fig. 2, the initial drop in activity is proportional to methanol concentration in solution. Although there is a continuous deactivation in the presence of methanol, there is a rapid initial deactivation rate, which decreases with time. Therefore, the displacement observed in the SCM would be the consequence of aggregation not directly related to deactivation, while the denatured enzyme would result in a higher fluorescence intensity at the same SCM, as observed at time zero, when the enzyme contacts the alcohol. If this is correct, the addition rate of methanol to precipitate the enzyme should be inversely proportional to the deactivation degree. In order to demonstrate this fact, experiments were performed precipitating the enzyme with 80% v/v methanol at two addition rates. As expected, the enzyme activity was completely lost when the addition of methanol was slow (1.2 ml/min of pure methanol added to obtain a final concentration of 80% v/v), while 67% was recovered in a rapid precipitation process (pouring 12 ml of methanol to obtain the same final concentration). Several hydrolytic enzymatic reactions are carried out in high concentrations of solvents and cosolvents in order to shift the reaction from thermodynamic to kinetic control. In other cases low water activities are used as a way to modify reaction selectivity. In the case of invertase we were able to obtain alcoholysis at a maximum conversion of 35% in 20% v/v methanol. This was possible by either reducing the reaction temperature or pH, avoiding the hydrolysis of the product, methyl-fructoside, and reducing sucrose hydrolysis. An alternative for a higher alcoholysis conversion is to increase the concentration of methanol. As already shown, the rapid aggregation of invertase in 80% v/v methanol does not deactivate the enzyme, however the flocs in 80% v/v methanol are not active when sucrose is added and must therefore be diluted to recover the activity.

The activity lost is also a function of temper-

ature: it is generally recognized that enzyme recovery from solution by precipitation must be conducted at low temperatures. However, a slow addition rate to favor homogeneity, has an adverse effect on invertase when precipitated with methanol.

Fluorescence emission results are consistent with the behavior of the enzyme (aggregation and deactivation). The fact that the activity is not recovered after dilution of the enzyme stored in methanol is due to the denaturating effect of methanol and not to aggregation, as shown also by the modification of the fluorescence spectrum after dilution of the aggregated enzyme. In effect, the original emission spectrum is not recovered so that the denaturated and aggregated enzyme, when diluted, gives rise to an inactive form with lower emission intensity.

If the methanol deactivation effect is not related to aggregation, invertase immobilization by covalent linking should not necessarily protect the enzyme from denaturation. In effect, the enzyme immobilized in alumina by covalent links was less stable than the soluble enzyme in the presence of 20% v/v of methanol, reducing its half life from 5.97 to 4.85 h. This is in agreement with the results obtained by Ulbrich-Hofmann and Selisko [5] who obtained invertase derivatives in alumina and in an aminomethyl polystyrene divinyl benzene copolymer activated with tricholorotriazine. It may be concluded that in order to obtain a stable invertase derivative, suitable for reaction with methanol a more robust structure is required. The covalent linkages of these immobilization techniques are not sufficient to avoid the initial unfolding observed by fluorescence studies.

In order to obtain a more stable form of the enzyme for reaction with methanol, whole *S. cerevisiae* cells were employed. If cells are used directly, methanol induces autolysis, liberating the enzyme with similar results to those obtained with the soluble purified enzyme. Therefore, the cells were crosslinked as described in Section 2. As shown in Table 1, this is the most stable form of the enzyme for reaction in the presence of methanol. Nevertheless this derivative is also sensible to methanol concentrations higher than 20% v/v. The half life is reduced from 19.7 h at 20% to 1.45 h in 40% v/v methanol, while for the free enzyme the half life is reduced from 5.97 to 0.16 h for all values at 40°C.

Table 1
Effect of methanol concentration on the stability of a whole cell invertase-crosslinked derivative

[Methanol] (%)	Half life (hours)	
	Free enzyme	Crosslinked derivate
0 (Control)	(months)	(months)
10	24.05	35.95
20	5.97	19.68
40	0.16	1.45
60	0.01	0.05

3.4. Inhibitory effect by methanol

It is important to point out that in the presence of methanol two reactions take place, so the kinetic model should consider alcoholysis and hydrolysis. We have already reported data concerning these reactions [6]. In Fig. 6 it is shown that when plotting initial reaction rates in the presence of low concentrations of methanol ($\geq$ 10% v/v), the alcohol also behaves as a mixed inhibitor with a $K_i = 3.2$ M. A mixed-type inhibitor affects both V_{max} and K_m values. The simplest mixed type inhibition model is one in which the enzyme–inhibitor complex has a lower affinity than the free enzyme for the substrate and the enzyme–substrate–inhibitor complex is nonproductive [12]. In this case, methanol may form a weak complex at the non-polar part of an invertase site, blocking the accessibility of sucrose and/or the diffusion of the products.

The results show that methanol effects on activity and stability of invertase can be differentiated in three types, according to the methanol concentration used: At methanol concentrations lower than 10% v/v, methanol behaves as a

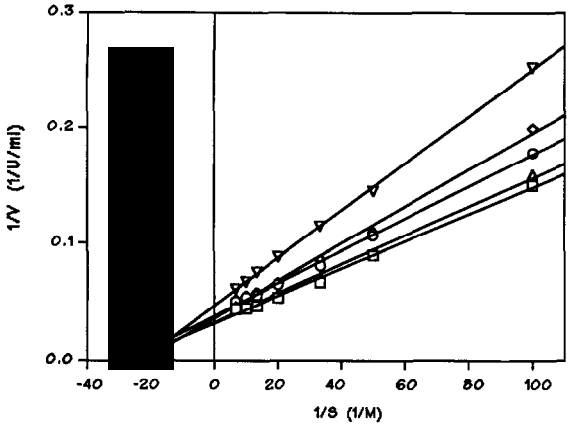

Fig. 6. Lineweaver–Burk plot of invertase kinetics in the presence of methanol at 40°C, pH 4.6, (□) control, (△) 0.247, (○) 0.741, (◇) 1.23 and (▽) 1.47 M.

mixed-type inhibitor and has a low effect on stability. At methanol concentrations higher than 20% v/v, the enzyme is inactivated in two steps: a rapid drop in activity proportional to methanol concentration upon contact of the enzyme with the alcohol, followed by a continuous deactivation but at a slower rate. Fluorescence emission studies demonstrate that methanol induces irreversible conformational changes related to deactivation. Finally, at methanol concentrations higher than 60% v/v, the enzyme precipitates from solution. Studies by GPC show that aggregation is reversible and is not the cause of inactivation.

As a conclusion invertase reactions with methanol carried out at concentrations of alcohol higher than 20% v/v result in low conversions due to enzyme deactivation, while low methanol concentration reactions should only take account of a mixed-type inhibition. Enzyme immobilization by the current procedures does not protect invertase from methanol deactivation and although aggregation at high methanol concentration protects the enzyme from denaturation, the aggregated form is not active in 80% v/v methanol. This suggests that a more effective way to produce stable derivatives for alcoholysis should combine intramolecular crosslinking followed by immobilization. Optimal conditions for methyl fructoside synthesis with a soluble enzyme may therefore be those found at around 20% v/v methanol combined with temperature and pH conditions favoring alcoholysis.

Acknowledgements

Financial support for this study was provided by DGAPA/UNAM grant No. IN-207293.

References

[1] J. Dordick, in: W. Harvey Blanch and S. Duglas (Eds.) Applied Biocatalysis, Vol.1 (Clark Marcel Dekker, New York, 1991) pp. 24–45.
[2] B. Selisko, R. Ulbrich-Hofman and A. Schellenberger, Biotechnol. Bioeng. 35 (1990) 1006–1010.
[3] D. Cavaille and D. Combes, J. Biotechnol. 43 (1995) 221–228.
[4] A. Straathof, J. Vrijenhoef, E. Sprangers, H. van Bekkum and G. Kieboom, J. Carbohydr. Chem. 7(1) (1988) 223–238.
[5] R. Ulbrich-Hofmann and B. Selisko, Enzyme Microb. Technol. 15 (1993) 33–41.
[6] M. Rodríguez, A. Gómez, F. Gónzalez, E. Barzana and A. López-Munguía, Appl. Biochem. Biotechnol. 59 (1996) 167–175.
[7] U.K. Laemmli, Nature 227 (1970) 680
[8] J. Sumner and S. Howell, J. Biol. Chem. 108 (1993) 51.
[9] H. Weetall, Methods Enzymol. 44 (1976) 134.
[10] E. Bismuto, D.M. Jamenson and E.E. Gratton, J. Am. Chem. Soc. 109 (1987) 2354–2357.
[11] R. Taussing and M. Carlson, Nucl. Acids Res. 11 (6) (1983) 1943–1955.
[12] I.H. Segel, Biochemical Calculations, 2nd Ed. (John Wiley and Sons, New York, 1975) pp. 261–266.