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A critical assessment of a viscometric assay for measuring *Saccharomycopsis fibuligera* α-amylase activity on gelatinised cassava starch

C.F. González[a], J.I. Fariña[a], L.I.C. Figueroa[a,b,*]

[a]*PROIMI, Av. Belgrano y Pje. Caseros (4000) Tucumán, Argentina*
[b]*Microbiología Superior, Facultad de Bioquímica, Universidad Nacional de Tucumán (4000) Tucumán, Argentina*

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Abstract

A viscometric technique for measuring *Saccharomycopsis fibuligera* DSM-70554 α-amylase on gelatinised cassava starch aqueous solutions was assessed. The selected conditions for working over a reliable viscosity measurement range involved a starch concentration of 5% (w/v) and a shear rate of 0.168 1/s. Viscometric assay involved the determination of the slope of the decrease in viscosity with time of the starch solution consequent on enzyme addition. Thereafter, a calibration curve was constructed by plotting the slopes, expressed in arbitrary viscometric units (AVU), versus the corresponding absolute activity (in IU) of either the commercial α-amylase from *Aspergillus oryzae* (up to 0.1 IU) or the *S. fibuligera* DSM-70554 α-amylase (up to *ca.* 0.4 IU). The amount of enzyme expressed in absolute terms produced different liquefying activities according to the α-amylase tested, emphasising the necessity of this correlation to be carried out for the particular enzyme being measured. In this work, a linear relationship and a very good correlation factor were achieved for the calibration of both amylases. Likewise, α-amylase activities determined according to the conventional reducing sugar determination and the colorimetric assay with iodine were proportional to those viscometrically obtained, both for *A. oryzae* and *S. fibuligera* α-amylase, validating conversions between different units. The viscometric assay herein described showed to be specific and sensitive and, after its calibration, it allows to convert α-amylase measurements in absolute units thus facilitating future comparisons.

Keywords: Saccharomycopsis fibuligera; α-amylase; Viscometric method

1. Introduction

Many microorganisms produce α-amylases and the ability of several yeasts to extracellularly secrete them has been previously referred [1]. In the extracellular amylolytic system of *Saccharomycopsis (Endomycopsis) fibuligera*, α-amylase (α-1,4-glucan 4-glucanohydrolase –EC 3.2.1.1) is the dominant enzyme and convert this strain in a commercially valuable tool for special biotechnological processes [2–4]. It catalyses the hydrolysis of α-D-1,4 glycosidic linkages of starch components (amylose and amylopectin), glycogen, and various oligosaccharides and exhibits low affinity for short chain oligomers [5]. Consequent on its action, formation of polysaccharides of lower degree of polymerisation takes place. Accordingly, the analysis of the amylolytic products and the distribution profiles versus time have allowed to establish the kinetics of α-amylase from *S. fibuligera* [6].

Since starch is one of the main biotechnological products from human history, different aspects of its enzymatic hydrolysis by α-amylases are still widely investigated by numerous scientists [1,6,7]. On the other hand, new active starch-degrading yeasts and mutants with increased amylolytic activity are permanently discovered, which stresses the requirement of reliable techniques for α-amylase determination and characterisation [8].

The botanical source is closely related to the chemical and functional properties of a starch, since it might influence critical characteristics such as granule size distribution, crystallinity or the chemical nature of the starch polymers. Nowadays, new genotypes of existing starch-rich crops are searched for study [9]. Among them, cassava constitutes a

* Corresponding author. Tel.: +1-54-381-4344888; fax: +1-54-381-4344887.

E-mail address: lifguero@proimi.edu.ar (Dr. L.I.C. Figueroa).

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promising source of starch and it is a staple crop of immense importance in the North of Argentina, particularly in the province of Misiones. In general, cassava constitutes a staple food for many countries and a source of raw material for agroindustrial development, and thus plays an important role in tropical agriculture [10]. However, cassava starch and its degradation by α-amylases from yeast origin have, up to now, been poorly investigated. Among the post-harvest technologies for cassava roots, the use of yeast amylolytic systems to introduce fermentation as a biotechnological tool for the improvement of protein content has been intended [11].

The difficulties for amylolytic activity determination are well reflected in the variety of analytical procedures so far devised. They are mainly of three types (in order of citation frequency): 1) assays measuring the increase in reducing power by means of reducing sugars determination; 2) measuring the stainability of residual starch by forming complexes with iodine; or 3) assays based on the decrease in viscosity of starch solutions during enzymatic reaction.

In this work, the α-amylase activity of crude enzyme preparations from *S. fibuligera* DSM-70554 grown with gelatinised cassava starch as the sole C-source was comparatively assayed by several common analytical procedures described in the literature. The aim of this work was: 1) to evaluate the sensitivity of different amylase tests for measuring enzymatic activities; 2) to convert these activities in absolute units in order to allow comparisons with results from different research groups and methods; and 3) to check the synthesised α-amylase activity on the same gelatinised cassava starch used for enzyme production.

2. Material and methods

2.1. Microorganism and culture conditions for α-amylase production

The α-amylase-producing yeast *Saccharomycopsis fibuligera* DSM-70554 is conserved in 30% glycerol at –20°C. For following experiments it was maintained by subculturing in YEPD agar slants containing (in g/liter): yeast extract, 10; peptone, 10; dextrose, 10; agar, 20. The inoculum was prepared by cultivating in 250-ml Erlenmeyer flasks with 50 ml of liquid YEPD pH 5.0. Incubation was carried out at 30°C and 250 rpm for 16 h.

For α-amylase production, the strain was cultured in a working volume of 200 ml in a 1000-ml Erlenmeyer flask. Cultivation was started by inoculating with a 16-h YEPD liquid culture so that the final yeast concentration in the flask was 0.1–0.2 g/liter biomass dry weight. The culture medium for α-amylase production contained (in g/liter): yeast extract, 10; $MgCl_2$, 0.6; $CaCl_2$, 0.1; cassava starch, 10. All components were dissolved in 0.2 M acetate buffer pH 5.5 before autoclaving. The medium was first heated for the gelatinisation of cassava starch and afterwards sterilised at 1 atm for 30 min. The following conditions were maintained throughout the fermentation: temperature, 30°C; pH, 5.5; agitation rate, 250 rpm.

After 24 h of cultivation, culture broth was divided into 50-ml aliquots which were centrifuged at 27,100 *g* and 4°C for 20 min. The supernatants were filtered through cellulose nitrate membranes (0.45 μm) and the filtrates, if not immediately assayed, were frozen and stored at –20°C until used for α-amylase determinations according to the methods herein described.

2.2. Starch and gel preparation for enzyme hydrolysis

Cassava starch (*Manihot esculenta*) from Misiones, Argentina, was obtained from a commercial source. Cassava starch suspensions were gelatinised by cooking the appropriate amount of polysaccharide dissolved in 10 mM acetate buffer pH 5.5 (for *S. fibuligera* α-amylase) or in 10 mM phosphate buffer pH 7.0 (for *A. oryzae* α-amylase). After stirring until homogeneous solutions were obtained, they were cooled to 40°C (storage temperature, T_S) and maintained at this temperature until viscosity measurements. Starch solutions were always used within 2 h of preparation. Keeping starch solutions at $T_S = 40$°C allowed to minimise retrogradation effects [12,13], as witnessed by the constancy in the rheological parameters consistency coefficient (*K*) and flow behaviour index (*n*) [14,15] estimated for different preparations of 5% (w/v) cassava starch solutions.

2.3. Enzymes

The first enzyme used for the calibration of the viscometric method was α-amylase (EC 3.2.1.1) type X-A from *Aspergillus oryzae* (A0273, Sigma Chemical Co., St. Louis, MO, USA), with an activity of 200 units/mg prot. and 40 units/mg solid. According to the manufacturer, one unit of enzyme (1 IU) is defined as the amount that liberates 1.0 mg of maltose from starch in 3 min at pH 6.9 and 20°C (following the Sigma quality control test procedure for the enzymatic assay of α-amylase (EC 3.2.1.1)).

The same definition and protocol were used for expressing the *S. fibuligera* α-amylase activity in absolute units, but optimal pH and temperature were corrected for this enzyme (see *2.4.1.*). This enzyme was used as a crude extract from the aliquots of supernatant from fermentation broth.

Both the α-amylase from *A. oryzae* and the crude α-amylase from *S. fibuligera* were comparatively assayed by the different available methods described below.

2.4. Assays for α-amylase determination

2.4.1. Activity in absolute units

It was determined according to the Sigma quality control test procedure for the enzymatic assay of α-amylase (EC 3.2.1.1).

Principle:

$$\text{Starch} + H_20 \xrightarrow{\alpha\text{-amylase}} \text{Reducing Groups}$$

(Maltose)

Conditions: T = 20°C, pH = 6.9, $A_{540\ nm}$, Light path = 1 cm –for *A. oryzae* α-amylase, and when measuring *S. fibuligera* α-amylase, conditions for the enzymatic reaction were changed to those which are optimal for this enzyme, i.e. T = 40°C, pH = 5.5.

Procedure: Soluble starch solution, 1% (w/v), was prepared by heating directly on a heating/stir plate the appropriate amount of starch dissolved in 20 mM NaH_2PO_4/6.7 mM NaCl buffer pH 6.9. In the Test tube containing 1 ml of this solution it was added 1 ml of enzyme solution and, after mixing by swirling, the mixture was incubated at the optimal temperature for the enzyme for exactly 3 min. The reaction was stopped by addition of 1 ml of the colour reagent solution (12 g sodium potassium tartrate tetrahydrate in 8 ml of 2 M NaOH plus 20 ml of 96 mM 3,5-dinitrosalicylic acid, all made up to 40 ml). A Blank tube contained starch solution and enzyme was added only after the colour reagent solution. The tubes were capped and placed in a boiling water bath for exactly 15 min. After cooling on ice to room temperature, 9 ml of distilled water were added and the tubes were mixed by inversion. The $A_{540\ nm}$ was recorded for both the Test and Blank samples and the milligrams of Maltose liberated were determined using a standard curve.

Calculations:

$$\text{Unites/ml enzyme} = \frac{(\text{mg of Maltose released})\ (\text{df})}{(1)}$$

df = dilution factor
1 = Volume (in ml) of enzyme used

2.4.2. Viscometric assay

Enzymatic hydrolysis was always performed with freshly gelatinised cassava starch (see *2.2.*). Viscometric determinations were performed with a Cannon LV2000 viscometer, equipped with a small sample adapter and a temperature controller unit (TCU). After a first screening for optimal conditions of measurement it was noticed that over the first 14 min of viscometric determination, no significant unspecific decrease (i.e. not related to enzymatic activity) in cassava starch viscosity was found.

Conditions of measurement were: TL-7 spindle, shear rate (γ) = 0.168 1/s, sample volume = 10 ml, cassava starch concentration = 5% (w/v), pH and measurement temperature (T_M) according to the tested enzyme (see below), overall time of measurement = 14 min (coincident with the time of enzymatic reaction). For viscometric determinations with *A. oryzae* α-amylase the volume of added enzyme was 10 μl, and for the crude α-amylase from *S. fibuligera* the volume was 500 μl. In all assays, the mixture resulting after enzyme addition was rapidly homogenised with a fine glass rod, and viscosity measurements were immediately continued. The timing for reading considered t = 0 as the time when enzyme was added to the substrate. The starch concentration was considered to be not limiting for the amylolytic reaction since the reported K_m values for starch of many amylases are within a range of 0.35 to 4.7 mg/ml [16,17].

2.4.3. Reductometric colorimetric assay

Amylase activity was determined by measuring the reducing sugars (RS) released from soluble starch using the alkaline 3,5-dinitrosalicylate reagent (DNS) [18]. The reaction mixture contained 100 μl of enzyme sample and 300 μl of 1% (w/v) soluble starch (Merck) in the corresponding buffer (see below). The blank sample contained no starch and the control sample contained no enzyme. After 10 min of incubation at the optimal temperature for the enzyme (see below), the reaction was stopped by adding 770 μl of DNS reagent. Colour was developed by heating in a boiling water bath for 10 min and then, the reducing groups were determined at 590 nm using a calibration curve with glucose as standard.

2.4.4. Colorimetric assay with iodine

It was based on the reduction in blue value resulting from the enzymatic hydrolysis of starch. The reaction mixture contained 300 μl of 1% (w/v) soluble starch (Merck) in the corresponding buffer (see below) and 100 μl of enzyme sample. The blank sample contained no starch and the control sample contained no enzyme. After 10 min of incubation at the optimal temperature for the enzyme (see below), the reaction was stopped by adding 500 μl of an iodine solution (10 mM I_2/10 mM KI). The resulting mixture was made up to a final volume of 20 ml with distilled water and the absorbance of the samples was measured at 550 nm [19].

For all assays in *2.4.2.*, *2.4.3.* and *2.4.4.*, the pH values were adjusted to the enzyme tested. Namely, starch solutions were prepared in 10 mM acetate buffer pH 5.5 for *S. fibuligera* α-amylase, or in 10 mM phosphate buffer pH 7.0 for *A. oryzae* α-amylase. Enzymatic reactions were performed at T = 40°C for *S. fibuligera* α-amylase, and at T = 25°C for *A. oryzae* α-amylase.

3. Results

The calibration of the viscometric assay was first carried out with commercial α-amylase from *A. oryzae*. Enzyme activity was expressed in arbitrary viscometric units (AVU) defined as the slope (in the linearity portion) of the line obtained by plotting log-log the ratio $\Delta(\eta,t_0 - \eta,t_x)$ against reaction time (Fig. 1), where η,t_0 is the apparent viscosity at time = 0 and η,t_x is the apparent viscosity at time t_x of the reaction [20]. Alpha-amylase activities expressed in AVU

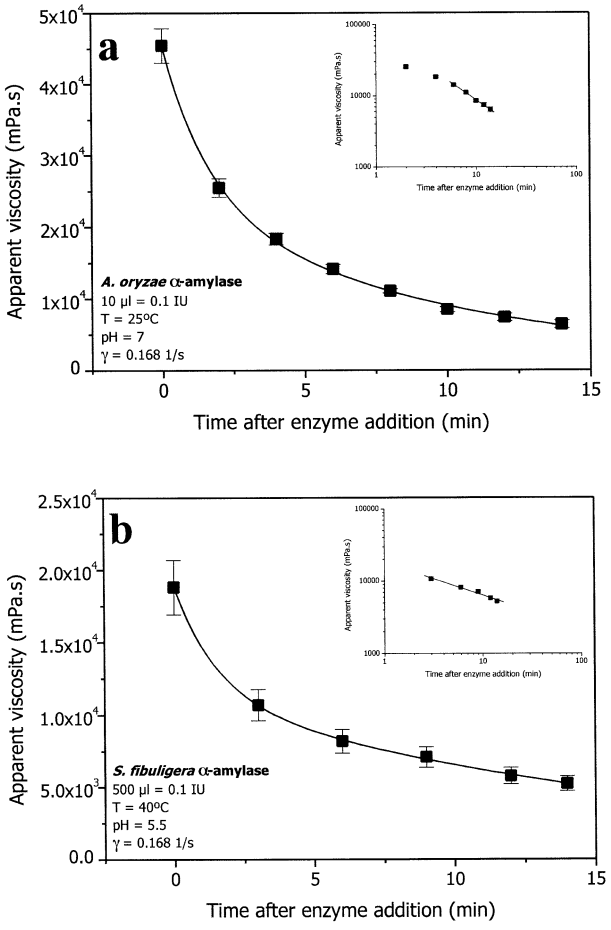

Fig. 1. Liquefying activity of α-amylase on cassava starch and determination of activity expressed in AVU. (a) *A. oryzae* type X-A α-amylase. (b) *S. fibuligera* DSM-70554 α-amylase. For further conditions of measurement see *Material and methods.* Curves corresponding to four independent determinations.

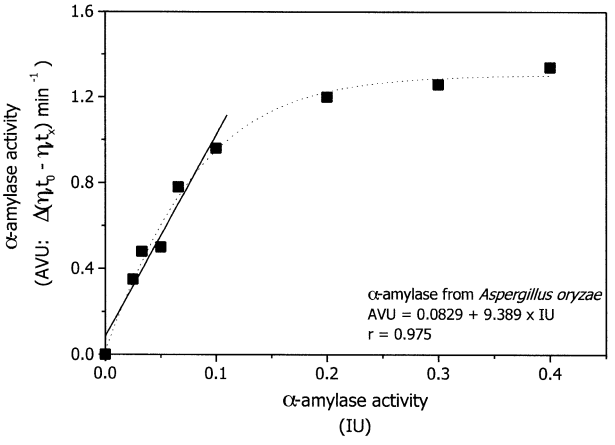

Fig. 2. Calibration curve for the viscometric assay with α-amylase type X-A from *A. oryzae.* T_S = 40°C, TL-7 spindle, sample volume = 10 ml, starch concentration = 5% (w/v), γ = 0.168 1/s, pH = 7.0, T_M = 25°C ± 0.5°C, time of measurement = 14 min, enzyme added = 10 μl.

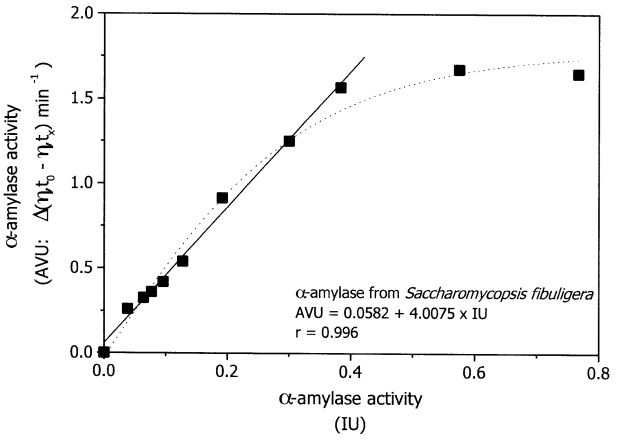

Fig. 3. Calibration curve for the viscometric assay with α-amylase from *S. fibuligera* DSM-70554. T_S = 40°C, TL-7 spindle, sample volume = 10 ml, starch concentration = 5% (w/v), γ = 0.168 1/s, pH = 5.5, T_M = 40°C ± 0.5°C, time of measurement = 14 min, enzyme added = 500 μl.

and in International Units (IU) were correlated (Fig. 2). Until 0.1 IU, a linear regression fitting could be applied in order to define an equation useful for the estimation of α-amylase absolute activity (in IU) (Fig. 2). In a similar way, a calibration curve was constructed for *S. fibuligera* α-amylase (Fig. 3), but in this case the linear regression fitting could be applied until 0.4 IU.

Amylolytic activity was also determined by quantification of the amount of reducing sugars released during enzymatic reaction (Figs. 4 and 5) and by measurement of the iodine-complexing ability of residual starch (Figs. 6 and 7). Both the reductometric and the colorimetric assay with iodine, were calibrated to convert α-amylase activity in international units (IU). Furthermore, equations for relating viscometric assay with these two other methods were defined for both enzymes (Figs. 4–7, insets).

Reductometric assay:

$$AVU = 0.1510 + 1.4428 \times \mu\text{moles RS/10 min (r} = 0.973)$$

for *A. oryzae* α-amylase

$$AVU = 0.0510 + 0.3225 \times \mu moles\ RS/10\ min\ (\text{r} = 0.996)$$

for *S. fibuligera* α-amylase

Colorimetric assay with iodine:

$$AVU = 0.4372 + 8.4240 \times \Delta OD_{550\ nm}\ (\text{r} = 0.950)$$

for *A. oryzae* α-amylase

$$AVU = 0.0908 + 1.7281 \times \Delta OD_{550\ nm}$$

(r = 0.996) for *S. fibuligera* α-amylase

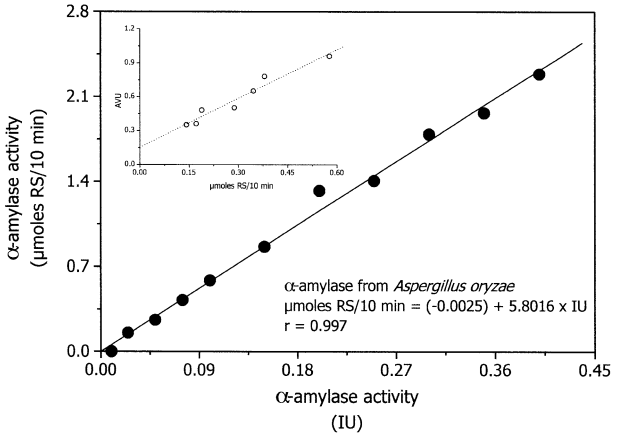

Fig. 4. Calibration curve for the reductometric colorimetric assay with α-amylase type X-A from *A. oryzae*. Inset: correlation with the viscometric assay. Enzymatic reaction conditions: T = 25°C, pH = 7.0 (for further details see *Material and methods*).

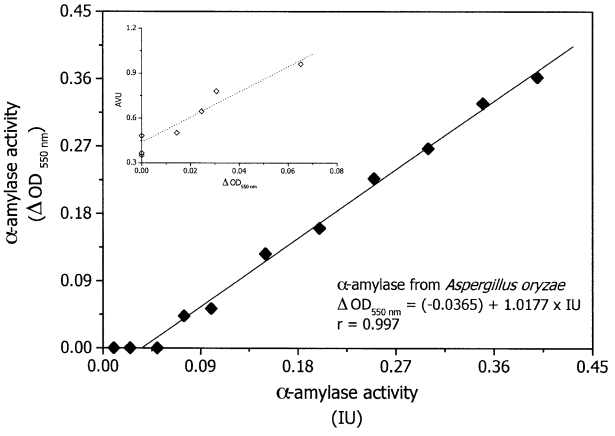

Fig. 6. Calibration curve for the colorimetric assay with iodine using α-amylase type X-A from *Aspergillus oryzae*. Inset: correlation with the viscometric assay. Enzymatic reaction conditions: T = 25°C, pH = 7.0 (for further details see *Material and methods*).

4. Discussion

Studies concerning amylolytic yeasts might be focused to many different aspects from biotechnological, biochemical and biologic points of view. Consequently, results from so diversified areas are often difficult to be objectively compared. Therefore, the assessment of analytical procedures to determine α-amylase activity is critical to permit direct comparisons between results from different research groups.

Reducing power determinations have the disadvantage to simultaneously measure *endo*- and *exo*-amylolytic activity and the interference of reducing sugars contained on the enzyme sample and/or generated by *exo*-enzymes is also a frequent drawback. Moreover, the possibility of alkaline hydrolysis during reductometric assays could eventually cause an unspecific increase not related to the enzyme activity. Furthermore, these methods might be not stoichiometric since equimolar quantities of maltodextrins of different sizes do not have equal reducing powers to alkaline 3,5-dinitrosalicylate [20–22].

Colorimetric methods with iodine are based on the stainability of residual starch and its hydrolytic products after enzyme digestion. The maltodextrins consequent on the amylolytic activity have not equal complexing ability with iodine. It is well reflected in the variety of obtainable iodine stains before reaching the achroic point, depending on the maltodextrin composition of the sample [22–24]. Therefore, the use of this technique for α-amylase activity determination might be a source of misleading results.

These two methodologies above discussed have been frequently recommended for comparative purposes, for example, when studying enzyme activities at different substrate concentration, pH or temperature. The viscometric method is a third known technique which was previously referred as the most sensitive though too time-consuming to

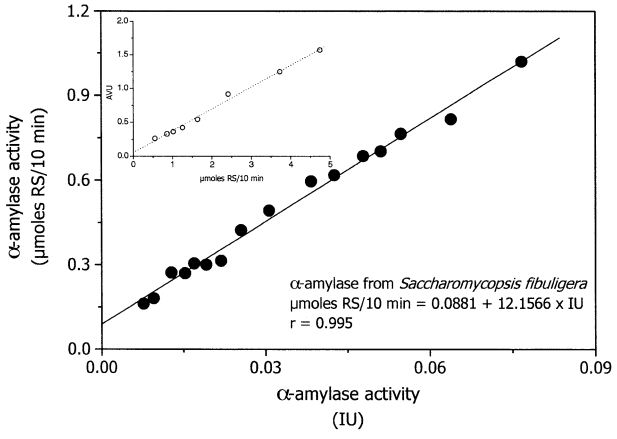

Fig. 5. Calibration curve for the reductometric colorimetric assay with α-amylase from *S. fibuligera* DSM-70554. Inset: correlation with the viscometric assay. Enzymatic reaction conditions: T = 40°C, pH = 5.5 (for further details see *Material and methods*).

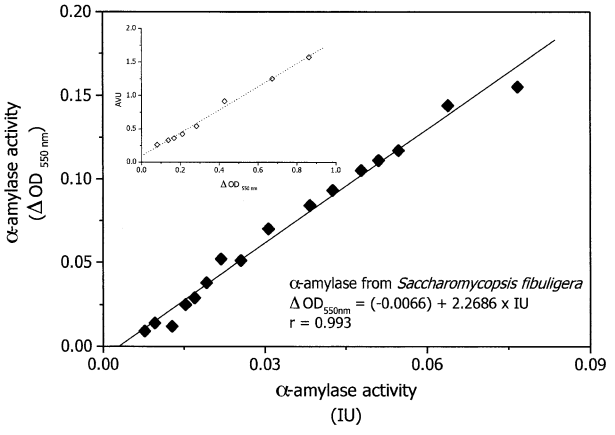

Fig. 7. Calibration curve for the colorimetric assay with iodine using α-amylase from *S. fibuligera* DSM-70554. Inset: correlation with the viscometric assay. Enzymatic reaction conditions: T = 40°C, pH = 5.5 (for further details see *Material and methods*).

be used as a routine method but otherwise useful when comparing results from different sources [22]. One of the main advantages of viscometric assays resides in the lack of interference for reducing sugars, which make them suitable for monitoring α-amylase activity in a variety of culture fluids.

The comparison of results reported by different authors is made difficult by the use of different substrates and the different expressions for α-amylase activity, depending on the measurement method used. On this context, the search for a direct relationship between arbitrary units and international units would allow objective comparisons, as it was investigated in the case of *endo*-cellulase activities [21].

In this work, α-amylase activities viscometrically determined were proportional to those obtained by the conventional methods frequently used (reductometric and colorimetric with iodine), as evidenced by the constancy for the different conversion factors and for the both enzymes tested (Figs. 4–7, insets). By means of the construction of suitable calibration curves, all the α-amylase activities could be expressed in IU.

When analysing the calibration curves for viscometric determinations of α-amylase (Figs. 2 and 3), and comparing results between *A. oryzae* and *S. fibuligera* α-amylase, it was found that the *endo*-amylase activities of these enzymes were different from each other. The amount of enzyme expressed in absolute terms produced different liquefying activities according to the α-amylase tested, emphasising the necessity of this correlation to be carried out for the particular enzyme being measured. This fact was evidenced by the slopes corresponding to the correlations AVU *vs.* IU for these enzymes, it is 9.3892 for *A. oryzae* α-amylase and 4.0075 for *S. fibuligera* α-amylase.

Considering these results, some speculations with respect to the mode of action of the measured enzymes might be taken into account. *A. oryzae* α-amylase seems to posses a greater *endo*-amylolytic activity than the *S. fibuligera* α-amylase which determines a higher number of glycosidic linkages are split per IU of enzyme, and consequently, starch solution viscosity is much rapidly decreased (Fig. 1). Accordingly, this reasoning could explain why the appropriate range of measurement for amylolytic activity results to be narrower in the case of *A. oryzae* α-amylase than in *S. fibuligera* α-amylase (up to 0.1 and up to 0.4 IU respectively). Furthermore, viscometric determinations were also useful to demonstrate the great ability of *S. fibuligera* α-amylase as a gelatinised cassava starch-degrading enzyme.

5. Conclusion

Despite different α-amylase determinations may be adopted, provided that they all yield a linear response with enzyme concentration, only assays which are specific, sensitive, and free of confusing interferences are suitable. That is the case of viscometric method, whose use would be recommended when comparisons are to be made with α-amylases from other sources. A frequent drawback of this method is the inability to express the enzyme activity in absolute terms or the number of catalytic events in unit time (IU). In this work this was conveniently solved by the construction of a calibration curve which became the method applicable for comparative purposes. This fact would be crucial when comparing our results with those elsewhere reported.

On the other hand, it has been previously reported that *S. fibuligera* produces an amylolytic complex that consists of an α-amylase, a glucoamylase, and a maltase. The reductometric α-amylase determination is often reported to measure glucoamylase activity. Likewise, the amylase iodine colour determination, often referred to as an α-amylase method, gave similar results. Thus, the task of differentiating α-amylase and glucoamylase is difficult because both enzymes have activity in either assay [4].

The viscometric assay also evidenced the high amylolytic activity of *S. fibuligera* DSM-70554 against gelatinised cassava starch, demonstrating that this poorly exploited polysaccharide could be utilised not only for inducing α-amylase production, but also for other biotechnological processes consequent on the release of more easily assimilable oligosaccharides.

Acknowledgments

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