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Production and Characterization of Amylases from *Zea mays* Malt

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ABSTRACT

In this work the α and β-amylase enzymes were obtained from maize (Zea mays) malt and were biochemistry characterized. A germination study to obtain the maize malt with good amylase activity was made. The maize seeds were germinated in laboratory and the enzymatic activity was measured daily. Activity dependence to germination time were fitted to an exponential model ($A=A_0e^{\mu t}$), which showed that the behaviour of enzymatic activity in the germination process was similar to the growth of the microorganism. Its model could be applied to describe the mechanism of α-amylase production for each maize varieties and others cereals. Maize malt characterization showed that α and β-amylase had optimal pH between 4-6.5, optimal temperature 50 and 90°C, and molecular weight of 67.4 and 47.5kDa, respectively. This work contributed with the advances in biotechnology generating of conditions for application of a new and of low price amylases source.

Key words: Maize seed, malting; α and β-amylase, biochemistry characterization, germination model

INTRODUCTION

The maize (*Zea mays*) is an agricultural product widely distributed in Brazil, and is commercialized natural form or with minimal processing to reduce the cost. Obtaining amylases from maize malt could add the price to the maize culture (Biazus et al., 2005a; Biazus et al., 2006; Ferreira et al., 2007). Amylases have several applications in industrial processes, essentially in the starch hydrolyse; and are used in food, pharmaceutical and textile industries (Biazus et al., 2005a; Dixon and Webb, 1971, Nirmala and Muralikrishna, 2003; Severo Júnior et al., 2007). Anybode costs U$ 1,500/gram of purified protein (Sigma, 1996).

α-Amylase (EC 3.3.1.1) is an extracellular enzyme which is widespread among higher plants, animals and microorganisms. It catalyses the hydrolysis of the α-D-(α-1,4) glucosidic linkage in starch components and related carbohydrates (Demirkan et al., 2005). It has isoelectric point of 5.4, excellent pH and temperature of enzymatic activity at 4.7 and 55°C, respectively, with molecular weight varying between 50-120 kDa (Dixon and Webb, 1971; Wiseman, 1987), but α-amylase from finger millet malt have molecular

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weight between 22-26 kDa (Nirmala and Muralikrishna, 2003), α-amylase from *T. lanuginosus* IISc 91 has 24 kDa (Nguyen et al., 2000), α-amylase from Lactobacillus manihotivorans LMG 18010^T, from *L. plantarum* L137, from *L. amylovorus* and *L. amylophilis* were of 135, 230, 140 and 100.kDa mol, respectively (Aguilar et al., 2000). This shows that α-amylase molecular weight is dependent from source (Aguilar et al., 2000; Biazus et al., 2005a; Dixon and Webb, 1971; Wiseman, 1987).

The β-amylase (EC 3.3.1.2) is an extracellular enzyme that hydrolyses amylopectin and glucogen, breaking each second α-1,4 bind, but activity at leaves no reductor boundaries of the molecules. The enzyme has excellent pH between 4.5 and 6.5, and 55°C, but its inactivated at temperatures above 60°C. Its isoelectric point is 4.8 and its molecular weight is between 20-50 kDa (Biazus *et al*., 2005a; Dixon and Webb, 1971; Wiseman, 1987).

In this work α and β-amylase enzymes were isolated from maize (*Zea mays*) malt and characterized. A germination study to prepare the maize malt with good amylase activity was also conducted.

MATERIALS AND METHODS

Reagents

An Amberlite IRA 410 ion-exchange resin from VETEC (São Paulo, Brazil) was used. Maize seeds were provided by EMBRAPA, Aracaju, Sergipe, Brazil. Sodium acetate, di-nitric salicylic acid (DNS), acetic and phosphoric acids, and potassium sodium tartrate ware provided by VETEC (São Paulo, Brazil). Potassium hydrogen phosphate, di-sodium hydrogen phosphate, Tris, SDS, comassie brilliant blue G-250 and chlorine acid were provided by MERK (Germany). Bio-Rad electrophoresis kit was proved by BIO-RAD (Germany).

Enzymes assays

Amylase activity was assayed according to Miller's method (Miller, 1959; Nirmala and Muralikrishna, 2003) and Wohlgenuth method, called as SKB method because it was modified by Sandstedt, Kneen and Blish (Biazus et al., 2005a, 2005b; Ferreira et al., 2007; Severo Júnior et al., 2007). Twenty milliliters of 2% (w/v) soluble starch at pH 4.8 (0.1 M acetate buffer) were hydrolyzed by 0.5 mL of enzyme sample at 30±2 C, for 10 min according to Miller method or for 1 h for SKB method. One unit of enzyme activity (U) was defined as μmol of glucose released per min under the assay conditions. Starch content that was dextrinized in one hour for one gram of amylase is called of SKB. DNS methos is not a specific method, it measures the activity of some hydrolytic enzymes, and however, SKB method is specific for *alpha*-amylase activity (Biazus et al., 2005a, 2005b, 2006; Ferreira et al., 2007; Miller, 1959; Nirmala and Muralikrishna, 2003; Severo Júnior et al., 2007). Total protein concentration was determined according to the dye binding method of Bradford (1976) with BSA as protein standard. The specific activity was calculated as activity for mass unit of protein (e.g. U/mg, SKB/mg, U/g or SKB/g).

Enzyme obtaining from maize malt

Maize seeds were selected, weighed and washed. These seeds absorbed between 40 and 45% (w/w) of the moisture and germinated in the laboratory at room temperature and pressure (27 °C and 1 atm). Enzymatic activity was measured daily. The maize malt was dried at 54°C for 5 h and stored at 5°C (Biazus et al. 2005a, 2005b, Nirmala and Muralikrishna, 2003; Malavasi and Malavasi, 2004; Santana, 2003). Crude samples, used in expanded bed chromatography, were obtained by mixing of 2 g of maize malt with 0.05 M phosphate buffers (pH 7) until 100 mL (Biazus et al., 2006, Santana, 2003).

Enzyme characterization

The optimum pH of the enzyme was determined measuring its activity, for 10 min in the pH range of 3.0-10.0 at 30°C ± 2°C, using 5 mL of distilled water and 20 mL of a 2% (w/v) soluble starch solution in the following buffers: 0.1M acetate (pH 3.0-5.0), 0.1M phosphate (pH 5.5-7.5) and 0.1M ammonium (pH 8.0-10). The optimum temperature was obtained measuring its activity, for 10 min at temperatures between 10 and 95°C ± 2°C, using some soluble starch solution in 0.1M acetate buffer at pH 4.8 (Aguilar et al., 2000; Ben Ali et al., 2001; Duedal- Olsen et al., 2000; Mohamed 2004; Wanderley et al., 2004). Initial rates of starch hydrolysis were determined at various substrate concentrations (0.01-20 mg/mL). The kinetic constants K_m and V_{max} were estimated by

Lineweaver-Burk method. SDS-PAGE was performed on mini-PROTEAN II cell (Bio-Rad, USA) with 12 % acrylamide gel, using buffer protein molecular weight marker (Aguilar et al., 2000; Ben Ali et al., 2001; Duedal- Olsen et al., 2000; Mohamed 2004).

Amylases purification

Expanded bed adsorption: EBA step was carried out at 22°C±2°C and pH 7. 2 g of ion-exchange resin was used; the fixed bed height was of 4.2 cm. Adsorbent bed was pre-equilibrated at 8 cm of expansion bed height, with 0.05 M phosphate buffer at pH 7 and flow rate of 14 mL/min. Five milliliter of maize malt crude was loaded in column bottom for promoting the adsorption of enzymes on Amberlite IRA 410 ion-exchange resin. The elution was done with 0.25 M NaCl at 14mL/min in descendent flux. The activity and the protein content were measured from time to time during the adsorption, washing and elution periods (Biazus et al., 2006; Kalil, 2000; Santos, 2001).

Expanded bed adsorption Column: EBA column used in the present work is shown in Figure 1. The glass column was 1x30 cm with an adjustable piston and feed flow inlet at the bottom of the column and a product flow outlet at the top. Sixty mesh plates at the feed inlet and at the product outlet were used to avoid loss of adsorbents particles. A ruler was placed at the side of the column for measurement of bed height (Biazus et al., 2006; Kalil, 2000; Santos, 2001).

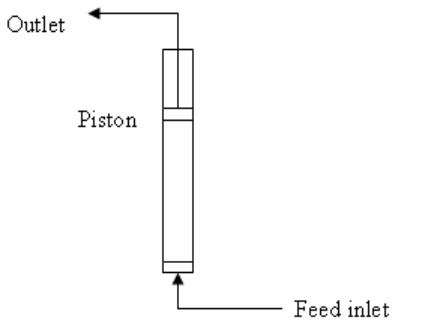

Figure 1 - Scheme of the EBA column.

Fractional precipitation: was carried out in 10 mL centrifuge tubes, with pH and temperature of precipitation medium adjusted at 7 by 0.05M phosphate buffer and at 20°C±2°C by water bath, respectively. Ethanol was added to the samples purified until 50-80% (v/v) of fractional concentrations were obtained. This mixture was agitated and centrifuged at 4000rpm and 4°C for 15min (Cortez and Pessoa Jr., 1999; Hilbring and Freitag, 2003). Total protein and enzymatic activity were determined in the supernatant (samples showed no precipitate) by Bradford and DNS methods.

RESULTS AND DISCUSSION

Germination modeling

Figure 2 showed that α-amylase activity into the maize seeds in germination process was a peak curve, while the fourth day was the optimal end time. This figure showed that enzymatic activity between the methods increased until fourth day, which was the optimal germination condition. SKB method showed the presence of α-amylase. From DNS and SKB curves showed that only α-amylase is produced by maize seed. The decrease of activity after fourth day was due to the production of inhibithors (e.g. hydrolysis products) or due reduction of starch concentration in maize seed.

It was seen that until the fourth day the curve was an exponential function. Thus, a model based on microbial growth could be proposed ($y = y_0 e^{b x}$). For analogy, the following nomenclature will be used:

$\boldsymbol{A}$ and $\boldsymbol{A_0}$ are enzymatic activity and initial enzymatic activity into the maize seeds (U/g), t is the germination time (day) and $\boldsymbol{\mu}$ is growth rate of enzymatic activity in the maize seeds (day^{-1}). Thus, the model could be expressed as:

$$A = A_0 e^{\mu t} \quad (1)$$

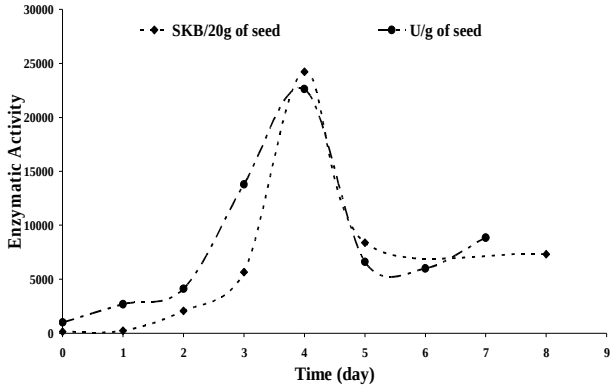

Figure 2 - Changing of *alpha*-amylase activity into the maize seeds in germination processes. Experimental data measured by Miller (●) and SKB methods (♦).

Figure 3 shows a germination scheme of monocotyledon seeds (e.g. maize) for the best understanding this phenomenon. Germination occurs after the radix breakes up seed tegument and it shows as a young radix. The energy needed for seed germination is provided by sugar from endosperm respiration. However, the embryo and starch are separated into seed and need an action of external forces to active its physiological functions. Water absortion is the external force that breaks the dormancy of seed (Biazus et al., 2005b; Malavasi and Malavasi, 2004; Santana, 2003).

Naturally, there is a small biological activity into maize seeds due to β-amylase enzyme. This enzyme hydrolyses the seed starch for producing maltose and supplements the seed cell with energy. Until the first day, was observed a low activity (Fig. 2).

When the water is absorbed by seeds, the embryo dissolves a substance, called gyberelic acid (GA), a vegetable hormone similar to the steroids. GA is transported with water into seed tissue until the aleuronic cytoplasm, and actives DNA nuclear. This DNA is a hereditary molecule and makes all proteins that the vegetable needs to survive. Its mechanism is not known (Biazus et al., 2005b; Santana, 2003).

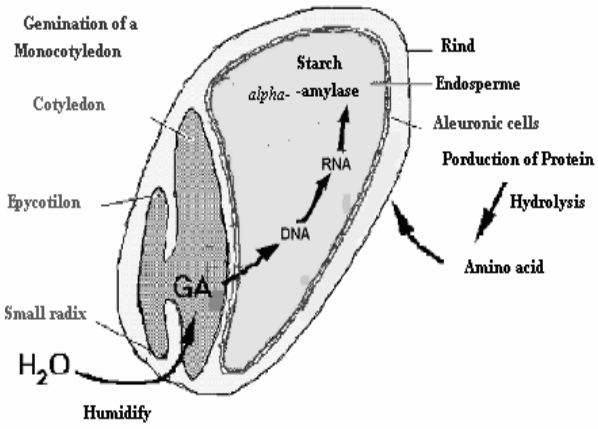

Figure 3 - Scheme of germination of a monocotyledon (maize seed). Source: Santana (2003).

The genes are transcripts and the informations are recorded in DNA. Aleuronic cells make discard copies of this DNA, called messenger RNA. This process of coping is called transcription. RNA is transported until the cytoplasm of aleuronic cell, joining it to the ribosome for beginning the process of making a specific protein. This process is called protean synthesis or translation. In this, the ribosome examines the information from the sequence bases of RNA (Biazus et al., 2005b; Santana, 2003).

The transporter RNA stores the specific amino acids that will be put in specific positions specified in the messenger RNA and their amino acids join in sequence determined by the ribosome. The property new protein will be determined by it amino acid sequence. In this case, the critical protein making is the α-amylase. This protein generates an enzyme of great importance for food industries. This occurred on second day until forty day; in present case, an exponential production of α-amylase enzyme, is shown in Figure 2 (Biazus et al., 2005b; Santana, 2003).

With these data, an exponential model was fit been fitting (R^2 = 0.981) at 95% of confidence level, and their parameters were found as seen in Figure 4.

The μ values were 0.7879 day^{-1} and 1.3833 day^{-1} and A_0 values were 1053.2 U/g and 4.576 SKB/g of maize seeds, respectively for DNS and SKB methods. Thus, the A_0 could be associated to the enzymatic activity of maize seeds before germination, or β-amylase activity (Malavasi and Malavasi, 2004). The parameter dependence of activity to germination time was proved by Biazus et al. (2005b) and Santana (2003). Equation 1 is a kinetic model of activity production into maize seeds. The model can be applied to describe the mechanism of α-amylase production for each maize varieties and the α-amylase growth in several cereal seeds.

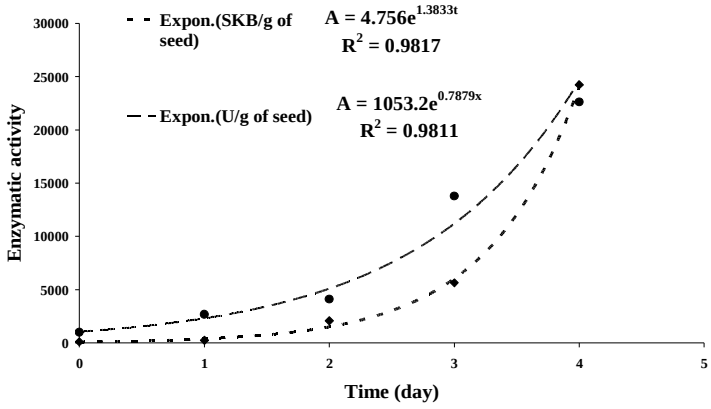

Figure 4 - Model evaluation used for simulating of increasing of the enzymatic activity of maize seeds in germination process. Experimental data measured by Milles (•) and SKB methods (♦) and, (--) and (- -) are their fitting model to data, respectively.

Biochemistry characterization

Figure 5 showed that α-amylase kept 80% of activity in a pH range between 4.0 and 6.5. This was similar to the literatures reports, as the pH optimum of most of α-amylase have been reported between pH 4.5-6.5 (Dixon and Webb, 1971; Mohamed, 2004; Reguly, 1996; Wanderley et al., 2004; Wiseman, 1987). However, there are α-amylases with pH optimum in basic range as from *Spodoptera frugiperda*, *S. littoralis* (pH 9.6) and *T. gondii* (pH 8.4) (Mohamed, 2004).

Figure 6 showed two peaks that indicated more than one enzyme into maize malt. It was probable that the first enzyme, at 50°C, was the β-amylase and second enzyme, at 90°C, was the α-amylase, as according to the previous reports they have optimal temperature about 55°C and above 70°C, respectively (Dixon and Webb, 1971; Wiseman, 1987). This thermal stability is a good factor to justify the use of this malt in industrial processes.

Literatures report the optimum temperature of optimal activity at 40°C for α-amylases from H.

bacteriophora, A. suum and S. litorallis; 50°C for α-amylases from C. flavus, S. alluvius ATCC 26074, L. kononenkoae and C. antarctica CBS 667 (Wanderley et al., 2004); 65°C for α-amylases from T gondii (Mohamedi, 2004); 55°C for α-amylases from L. Manihotivorans (Aguilar et al., 2000); and 70°C for α-amylases from T. lanuginosus (Nguyen et al., 2002). Generally, 70°C or higher is considerate as the optimum temperature of the most of the α-amylases (Biazus et al., 2005; Dixon and Weeb, 1971, Wiseman, 1987; Reguly, 1996).

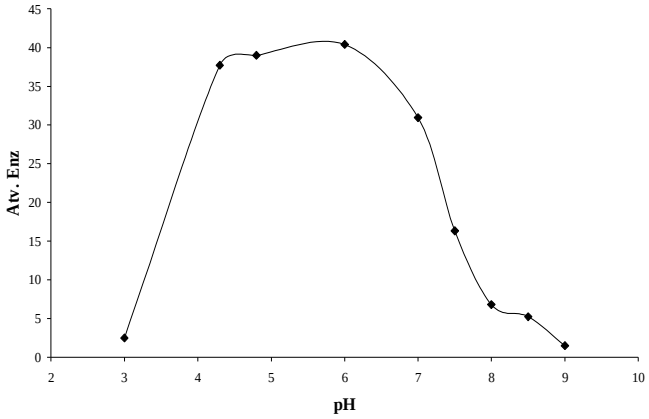

Figure 5 – pH effect on the activity of amylases from maize (*Zea mays*) malt.

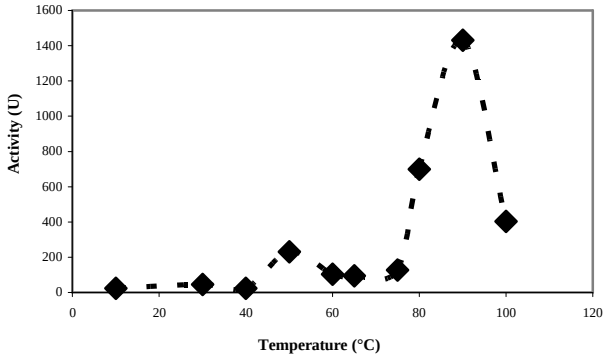

Figure 6 – Temperature effect on the activity of amylases from maize (*Zea mays*) malt.

By the kinetic data of starch hydrolysis by amylases from maize (*Zea mays*) malt, showed in Figure 7, it was possible to calculate the K_m, and V_{max} constants, by Lineweaver-burk method, equation 2. Their values were 7.69 10^{-2} g/L and 7.69 10^2 g/L.min (2.48 U/mg), respectively.

K_m constant was 69-186, which was lower than finger millet amylases (5.3-14.3 g/L) and it was lower than the α-amylase from species of granivorous coleopterans (0.061-0.42 mg/mL) and lepidopterans (0.037-0.38 mg/mL) (Nirmala and Muralikrishna, 2003), from *A. suum* (0.483.33 mg/mL), *H dromedarii* (10 mg/mL), *H. bacteriophora* (6.5 mg/mL) and *L manihotivorans* (3.44 mg/mL) (Aguilar et al., 2000). V_{max} value of maize malt amylases was 2.1-2.6 greater than finger millet amylases ($4.76.10^3$-$5.88.10^3$ U/mg) (Aguilar et al., 2000). This value was higher than other amylases (Aguilar et al., 2000, Nirmala and Muralikrishna, 2003)). It showed that amylase from maize malt had high affinity to the starch and this factor was very important for its industrial application (Nirmala and Muralikrishna, 2003).

$$V_0^{-1} = 1.3.10^{-3} + 1.10^{-4}[S]^{-1} \quad (R = 0.9915) \qquad (2)$$

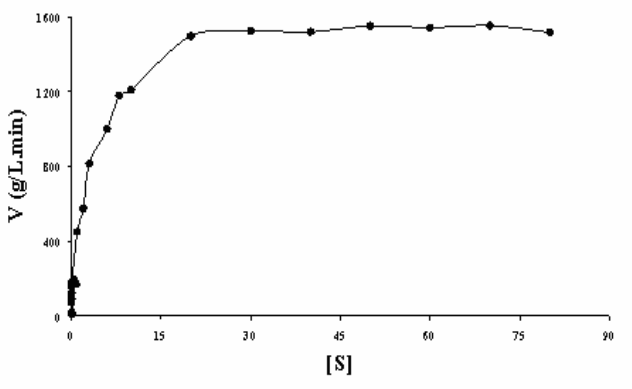

Figure 7 - Starch hydrolysis kinetic by amylases from maize (*Zea mays*) malt at pH 4.8 and 30°C.

Amylases capture

Figure 8 shows the purification of amylases from *Zea mays* malt by expanded bed adsorption. Analysis of this figure showed that the phosphate buffers and Amberlite IRA 410 were good medium for amylase adsorption due to lower interaction between the resin and ions from buffer. According to Biazus et al. (2006), the binding capacity of Amberlite IRA 410 ion-exchange resin at two expansion degrees (8 cm of bed height) was the best system for amylase recovery by the expanded bed chromatography in Amberlite IRA 410 ion-exchange resin. It could be associated with the presence of Ca^{2+} in α-amylase and Mg^{2+} in β-amylase structures, given their electro-negative conformations, which this enhanced their capture by resin actives sites (Biazus et al., 2006; Dixon and Webb, 1971; Kalil, 2000; Wiseman, 1987).

In a second step, the proteins eluted from EBA process were precipitated by ethanol fractional precipitation at pH 7 and 20°C±2°C. The supernatant at 50-80% (v/v) of ethanol concentration showed that the majority of amylases were captured in liquid phase. Original medium (maize malt) contained starch and products of starch hydrolysis, which were amylase inhibitors and these inhibitors are not captured by the resin, or they were little captured and precipitated by ethanol. The activity recovery was more than 100% and the purification factor (~100) was better than Aguilar et al. (2000), Demirkan et al. (2005), Nguyen et al. (2002), Nirmala and Muralikrishna (2003), Santana (2003) and Zhi et al. (2005).

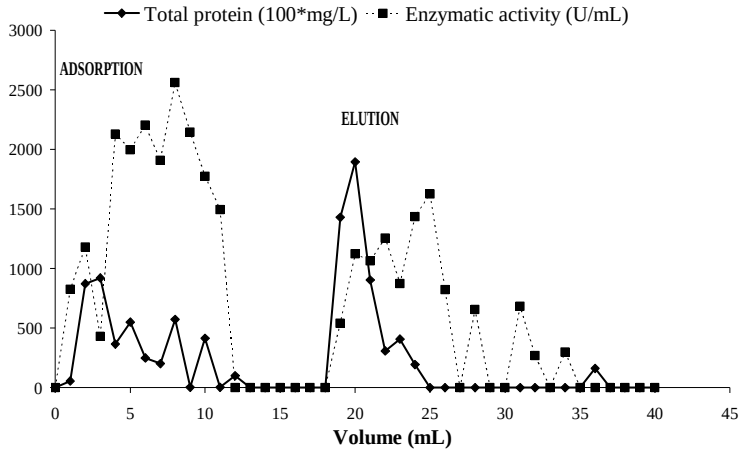

Figure 8 - Expanded bed chromatography curve of maize malt in phosphate buffer at pH 7 and 22°C and with elution using a 0.25M NaCl solution. Two expansion degree was used and fixed bed heigt was of 4.2 cm. Total protein (-♦-) and enzymatic activity (**..■..**).

After EBA process the protein concentration decreased about half of initial protein concentration of maize malt crude. According to Lucarine et al. (2005), it occurred due to the resolution of fractional precipitation to be raised to the decrease of protein concentration. Amylase inhibitor also is eliminated by ethanol precipitation and increase the purification factor.

Figure 9 is SDS-PAGE analysis of the samples of maize malt crude (***MM***), EBA eluted at pH 7 (***EEBA***) and supernatant of fractional precipitation (***EP***) of EBA eluted. This figure showed that there were adsorptions of the two proteins on the resins and that after frational precipitation, only one protein was remained.

It showed that the EBA and fractional precipitation step were very effective in amylase purification from *Zea mays* malt. The molecular weights in EBA eluted sample were 69.4 and 47.6 kDa, respectively, and in fractional precipitation 69.4 kDa. It was probable that the molecular weights could be of α and β-amylases, as several authors have cited that molecular weight of the first enzyme, commonly, was between 50-230 kDa and of the second enzyme between 20-50 kDa (Dixon and Webb, 1971; Wiseman, 1987).

According to literature, the molecular weight is dependent of α-amylase source; α-amylase from *Bacillus clausii* (Duedal-Olsen et al., 2000) and *H. bacteriophora* 47 kDa, from *A. suum* 83 kDa, from *H. dromedarii* 106 kDa (Mohamed, 2004), from *B. stearothermophilus* 59 kDa (Ben Ali et al., 2001) and from *L. manihotivoras* 135 kDa (Aguilar et al., 2000).

After fractional precipitation with ethanol, the remaining protein was α-amylase, which was according to maize germination study that showed that enzymatic activity of α-amylase into of maize malt was more than β-amylase. α-amylase activity increased exponentially and β-amylase activity was constant in germination process (Biazus et al., 2005b; Santana, 2003.

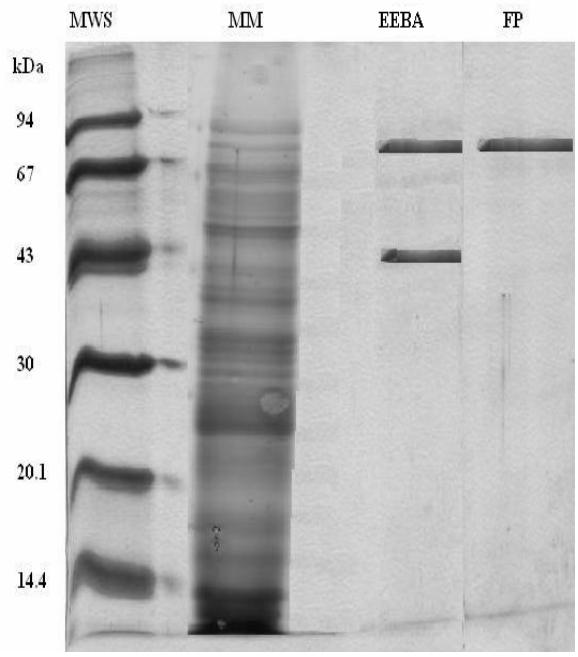

Figure 9 - Molecular weight determination by SDS-PAGE. ***MWS*** is molecular weight standard, it is compound of following proteins: phosphorylase b (94kDa), bovine serum albumin (67kDa), ovalbumin (43kDa), carbonic anhydrase (30kDa), trypsin inhibitor (20.1kDa) and α-lactoalbumin (14.4kDa).While ***MM*** is crude ample of maize malt, ***EEBA*** is EBA eluted sample and ***EP*** is fractional precipitate sample.

CONCLUSION

The maximum enzymatic activity in the maize seeds occured on the fourth day of germination and an exponential model, similar to microbial growth ($A=A_0e^{\mu t}$). This model could be used applied to describe the mechanism of α-amylase production for each maize varieties and others cereals.

Maize malt characterization showed that α and β-amylase had optimal pH between 4.0-6.5, optimal temperature 50 and 90°C, and molecular weight 67.4 and 47.5kDa, respectively.

This work contributed with the advances in biotechnology, showing the condition of apply an amylases isolated of a new source and of lower cost. This could add the price to maize culture.

RESUMO

Neste trabalho as enzimas α e β-amilases foram obtidas de malte de milho e depois foram caracterizadas bioquimicamente. Um estudo da germinação foi feito para obtenção do malte com boa atividade amilásica. A germinação ocorreu em escala laboratorial e a atividade enzimática foi medida diariamente. Um modelo exponencial do tipo $A=A_0e^{\mu t}$ foi ajustado a dependência do tempo de germinação com a atividade, mostrando que o comportamento da atividade enzimática no processo de germinação é semelhante ao crescimento de microorganismos. Este modelo pode ser aplicado para descrever o mecanismo de produção da α-amilase para cada variedade de milho e de outros cereais. A caracterização do malte de milho mostrou que as α e β-amilase têm pH ótimo entre 4,0-6,5, temperatura ótima de 50 e 90°C, e massa molar de 67,4 e 47,5 kDa, respectivamente. Este trabalho contribuiu com os avanços da biotecnologia gerando condições de emprego de uma nova e barata fonte de amilases.

ABBREVIATION

A	=	enzymatic activity
A_0	=	initial enzymatic activity
DNS	=	di-nitric salicylic acid
EP	=	ethanol precipitation
EBA	=	expanded bed adsorption
EEBA	=	eluted of expanded bed adsorption
GA	=	gyberelic acid
K_M	=	Michalis-Menten constant
MM	=	crude sample of maize malt
MWS	=	molecular weight standard
S	=	subtract concentration
SDS-PAGE	=	poly(acryl amid) gel electrophoresis using sodium dodecil sulfate as a denaturing agent.
SKB	=	Sandstedt, Kneen and Blish method
t	=	germination time
V_0	=	initial velocity of enzymatic reaction
V_{max}	=	maximum velocity of enzymatic reaction.
Greeks		
μ	=	growing rate of enzymatic activity into maize seed.

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