PRELIMINARY DATA REGARDING THE KINETIC PROPERTIES OF AN ALPHA-AMYLASE FROM *ROBINIA PSEUDACACIA* L. GERMINATED SEEDS

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Abstract: We have accomplished a partial purification of a α-amylase from germinated seeds of *Robinia pseudacacia* L. by affinity precipitation. The key element is the sodium alginate, a polymer that proved affinity for this enzyme, and also has the propriety to reversibly precipitate with Ca^{2+}. The enzyme binds to the alginate and the complex is precipitated with Ca^{2+}. The amylase activity is recovered by dissolving the precipitate in 1M maltose and precipitating the alginate alone by addition of Ca^{2+}. The enzyme has a molecular weight estimated between 50 and 65 kDa, an optimum pH between 5 and 6; it is inhibited by ammonium sulfate and activated by $CaCl_2$.

INTRODUCTION

α-Amylase (α-1,4-glucan 4-glucanohydrolase) hydrolyzes the α-1,4-glycosydic bonds inside the poliglucidic chains from starch, amylose, amylopectine, dextrins. This enzyme is widely spread both in animals, plants and microorganisms [1].

Inside the digestive tube of the animals, α-amylase participates in the depolimerization of the α-glucans from food, converting them to dextrins and finally to soluble oligoglucids. In plants α-amylase initiates the starch granules hydrolysis in vivo. Non-germinated cereal seeds contain very low levels of α-amylase. When the germinating process start, α-amylase biosynthesis in the aleurone layer increases dramatically. In the literature we have found many observations regarding the biosynthesis, structure and properties of α-amylase from cereals and other plants. The enzyme from ligneous leguminous species is less studied.

In this paper we report the partial purification and some properties of an α-amylase from *Robinia pseudacacia*.

MATERIAL AND METHODS

Plant material and germination. Locust tree (*Robinia pseudacacia* L.) seeds were collected from the „Anastasie Fătu” Botanic Garden of Iaşi. After sterilization, the seeds were subdued to a mechanical treatment, in order to destroy the structural integrity of the tegument, and to a heat treatment with 80°C-90°C water. The seeds were placed on Petri plates at room temperature and dark. The germination took place in 3 days.

Amylolytic Activity Assay. Activity of α-amylase was estimated using starch as substrate, according with the Noelting-Bernfeld method [3]. One enzyme unit (U) liberates 1 μmol of reducing sugar (calculated as maltose) per minute at 40°C and pH 5.6 from soluble starch.

Estimation of protein. Protein was estimated using Bradford dye-binding method, with bovine serum albumine as standard [4].

Partial purification of α-amylase. Partial purification of α-amylase was done using the method proposed by Gupta and colab.[5], which uses sodium alginate [7] as a support for enzyme binding. 20 g of plant material was homogenized and stirred with a total of 200 ml chilled acetone in cold for 2 hours. The dry acetonic powder obtained can be kept in the refrigerator, without any activity loses. 10 grams of this powder were used for enzyme extraction with 50 ml acetate buffer 0.5 M, pH 5.6 for 1 hour. The insoluble material was eliminated by centrifugation at 3000 rpm for 15 min.

The clear supernatant was subdued to a two step ammonium sulfate precipitation at 35% and 75%. After the last step, the precipitate is re-suspended in 10 ml acetate buffer and mixed with the 2% alginate solution (crude extract: alginate 2% 2/1). The final volume of the solution was made to 16 ml with acetate buffer. The enzyme-alginate complex is developed within 1 hour. The complex is then precipitated with $CaCl_2$ solution 1M to a final Ca^{2+}concentration of 0.6M. The precipitate was centrifuged at 3000 rpm for 15 min at 25°C, and then washed with acetate buffer containing 0.6 M $CaCl_2$. The complex is dissolved in acetate buffer containing 1M maltose (15 min). Without the calcium ions the polymer becomes soluble and the maltose induces the breakdown of enzyme-alginate complex. The alginate is eliminated from the solution by precipitation with calcium ions and centrifugation. The amylolytic activity of the supernatant was determined after dialysis of maltose against the buffer solution. The dialysis was done in two steps of 6 hours each; after every step the solution vas renewed. The solution obtained was used as enzyme source.

PAGE-SDS Electrophoresis. For purity verification and molecular weight evaluation PAGE-SDS electrophoresis was conducted, using 10% resolving gel and 5% stocking gel. As marker we used Pharmacy Biotech-53-212 kDa; staining of the gels was done with Coommassie Brillantblue R 250. Electrophoresis was carried out first at 15 mA-50 V, and then changed at 30 mA-150 V.

RESULTS AND DISCUSSIONS

Partial purification of α-amylase. Initially, we took samples from every purification step for protein and amylase activity assay. The ammonium sulfate proved to interfere with the determinations and we had to give up the samples with high sulfate concentrations: the supernatant after the precipitation with 35% ammonium sulfate and the supernatant with 70% sulfate. The acetone-washing step was necessary, as fractionation proved to be deficient without it (data not shown).

The evolution of the purification factor and yield along the purification steps are shown in the table:

Step	Volume (ml)	Total activity (U)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	40	60,88	14,907	4,083	100,00	1,00
Ammonium sulfate precipitation	9,7	39,432	3,725	10,585	64,77	2,59
After dialysis	29	32,838	0,79	51,271	53,94	12,55

We carried out the PAGE-SDS electrophoresis of these three samples and the photo of the gel is presented in figure 1. The brute extract showed 10 protein fractions with different electrophoretic mobility, which proves a good extraction. The sample taken after the ammonium sulfate precipitation shows thicker bands, but the number of the fractions is the same, although the total protein decreases from 14.9 mg to 3.725 mg and the purification factor increases.

The lanes 4 and 5 show that a great number of fractions have disappeared; from the initial ten we found only three. In the literature we have found reports that highly purified α-amylase appear as multiple fractions on PAGE-SDS, because the buffer used for electrophoresis do not have Ca^{2+}. During the electrophoresis some amylase loses this ion and migrates more rapidly [2]. It is not the case of our experiment, as the purification factor that we obtained shows that the solution contains some proteins without amylolytic activity. The explanation is that alginate shows affinity and for other proteins, like pectinase, phospho-lipase [6].

The estimated molecular weight is between 50 and 65 kDa, which agrees well with the literature [8].

Substrate specificity. We have studied the substrate specificity of the enzyme from both the crude extract and the post-dialysis solution upon 3 different substrates: soluble starch, glycogen and dextrin. The results are shown in figure 2.

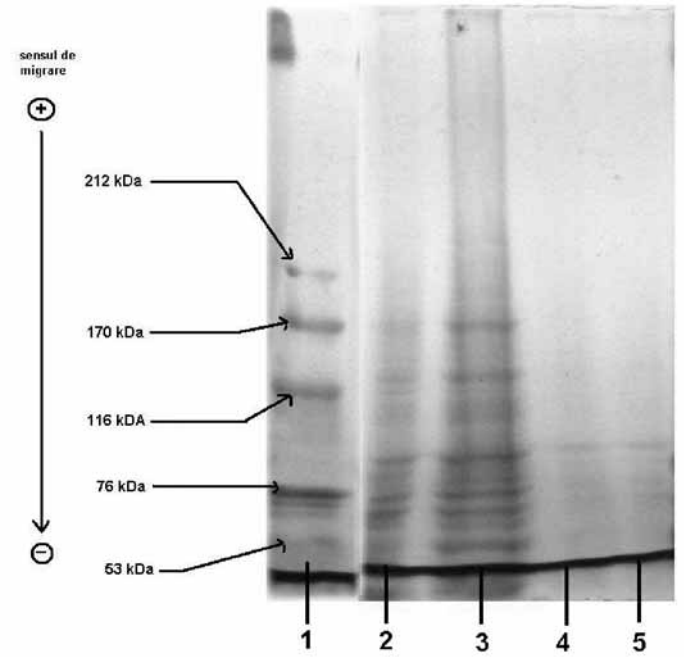

Figure 1. SDS-PAGE of samples taken in the purification steps. Lane 1-marker proteins, lane 2- crude extract, lane 3- ammonium sulfate precipitation step, lane 4 and 5- after dialysis

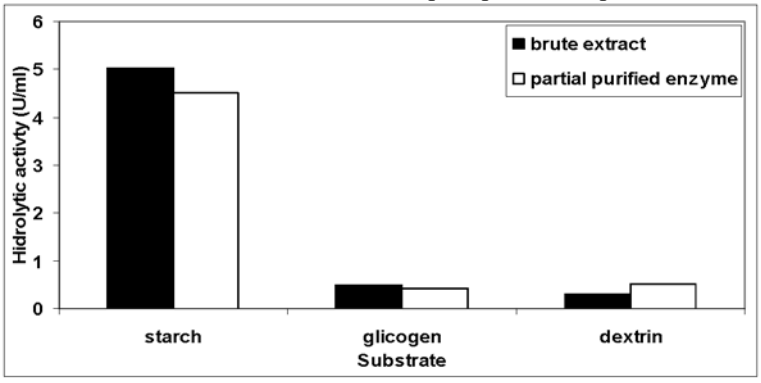

Figure 2. Hydrolytic activity of α-amylase from on different substrates

The enzyme behaves typically for a vegetal amylase. The small differences between the activity of the crude extract and the partial purified enzyme acting on starch can be explained in two ways. First, the solutions used as enzyme source are not equal regarding their amylolytic activity/ml. Second, the crude extract offers conditions much closer to those found in vivo (different types of ions, different biological molecules) that could stabilize the enzyme.

The influence of substrate concentration over the amylolytic activity. The dependence of the starch degrading rates upon starch concentration is presented in figure 3.

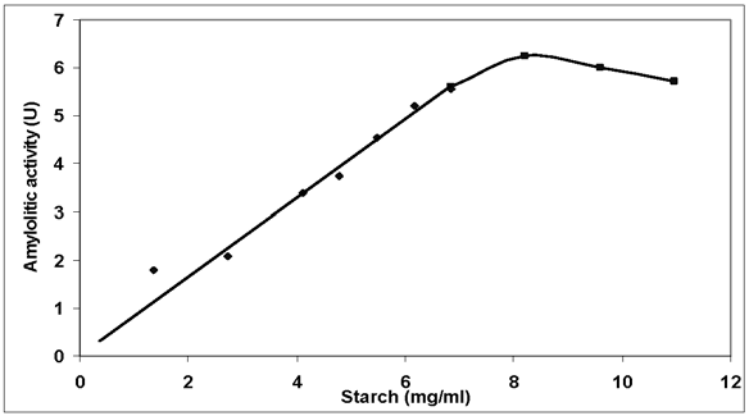

Figure 3. The relation between starch degrading rate and substrate concentration

The data shows that the enzyme does not behave typically according to a Michaelis-Menten kinetic, but more like as substrate-inhibited enzyme. This behavior has two explanations: for the hydrolases the water may be considered one of the substrates, the increasing of the starch concentration leading thus to the diminishing of water “concentration”. The second explanation is the starch degrading mechanism. According to the subsitus theory [8], the enzyme has several affinity sites for starch. By increasing its concentration a greater number of non-productive complexes are formed and the degrading rate decreases.

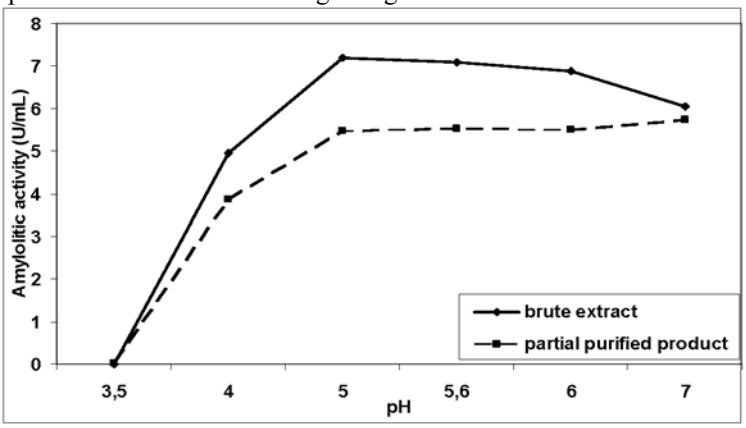

Figure 4. The influence of pH on the amylolytic activity of the α-amylase from both brute extract and partial purified product

The influence of pH and temperature on amylolityc activity. We have studied the influence ionization degree of the enzyme and substrate upon the starch degrading rate, and not the pH stability of the enzyme. The results are shown in figure 4.

For the enzyme from both sources, the optimum pH is on a rather broad plateau of 5-6 and, at low values, the starch degrading rate decreases dramatically: from 4,94 U/ml at pH 5 to 0.012 U/ml at pH 4 for the brute extract and from 3,851 U/ml to 0,036 U/ml for the partial purified enzyme. This would indicate the presence of some aminoacids with basic side chains in the catalytic site.

Concerning the temperature, we have studied its influence upon the degrading rate and not the enzyme termostability. The results are presented in figure 5.

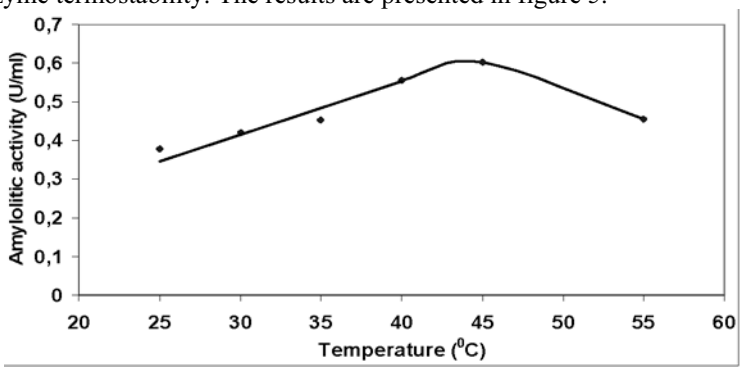

Figure 5. The influence of temperature on starch degrading rate.

The α-amylase from germinated seed of *Robinia pseudacacia* L has an optimum temperature of 45°C, similar to that from rice (45°C-50°C).

The influence of different inorganic ions over the activity of the α-amylase from *Robinia pseudacacia* L germinated seeds. During the purification process we observed that in the presence of ammonium sulfate at high concentration the enzyme activity decreases dramatically. We have studied the influence of different concentrations of the salt on the amylolytic activity of the enzyme. The results are shown in figure 6.

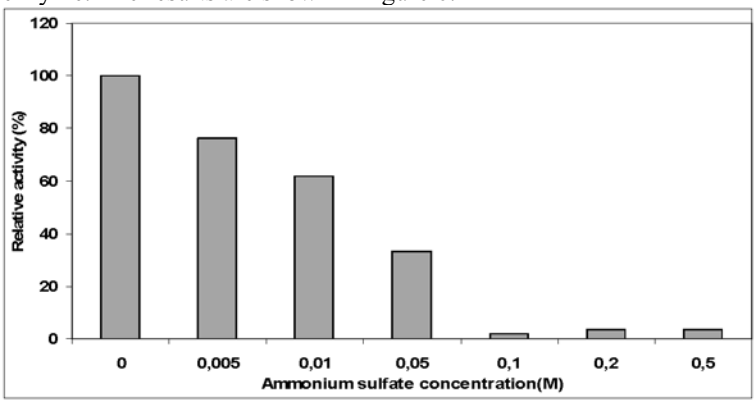

Figure 6. The inhibitory effect of ammonium sulfate on amylase activity

Concentration of only 0.005 M ammonium sulfate leads to a decrease of the amylolytic activity, from 3,165 U/ml for the control to 2,412 U/ml. By increasing the salt concentration to 0.05 M the enzyme activity decreases to about 33.33% of control. In the samples with high salt concentrations the activity decreases dramatically and we could observe a deficient development of the maltose specific color.

The calcium ion has a specific importance for the vegetal α-amylases because it has a thermostabilizing role. Without it the amylolytic activity disappears at 70°C because of thermodenaturation of the enzyme. We have investigated whether this ion has an activating effect on this enzyme. The results are presented in the following diagram (figure 7).

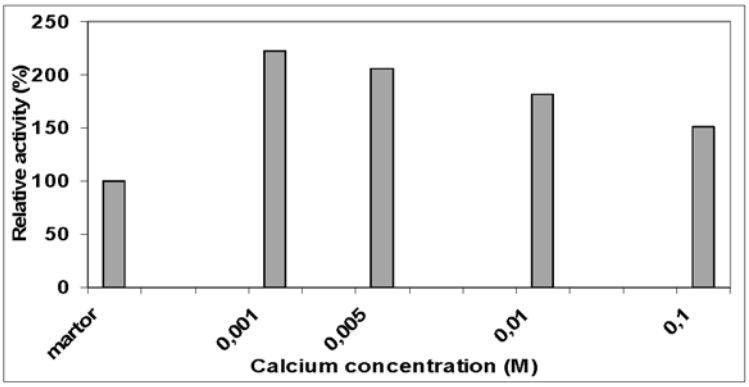

Figure 7. Activatory effect of the $CaCl_2$ on amylase activity

In the presence of the $CaCl_2$ 0.001 M the amylase activity increases to about 122% of the control value. Further increases of the salt concentration lead to a lost of enzyme activity. At 0.005 M $CaCl_2$ the activity is 4.359 U/ml and at 0.1 M becomes 3.213 U/ml. Although the activation effect decreases, it maintains above the control level values. In order to explain this effect, we have to consider both the Ca^{2+} and Cl^- ions.

CONCLUSIONS

The relative molecular weight of the α-amylase is between 50-65 kDa.
The enzyme is inhibited by its substrate at high concentrations
The optimum pH is a broad interval between 5-6.
The optimum temperature is found between the values of 40°C and 50°C.
$CaCl_2$ has an activating effect, especially at low concentrations.
Ammonium sulfate has an inhibiting effect, proportional with its concentration.

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