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Evaluation of Porcine and *Aspergillus oryzae* α-Amylases as Possible Model for the Human Enzyme

Mauro Marengo, Davide Pezzilli, Eleonora Gianquinto, Alex Fissore, Simonetta Oliaro-Bosso, Barbara Sgorbini, Francesca Spyrakis * and Salvatore Adinolfi *

Department of Drug Science and Technology, University of Turin, Via Giuria 9, 10125 Turin, Italy; mauro.marengo@unito.it (M.M.); davide.pezzilli@edu.unito.it (D.P.); eleonora.gianquinto@unito.it (E.G.); alex.fissore522@edu.unito.it (A.F.); simona.oliaro@unito.it (S.O.-B.); barbara.sgorbini@unito.it (B.S.)

* Correspondence: francesca.spyrakis@unito.it (F.S.); salvatore.adinolfi@unito.it (S.A.); Tel.: +39-011-670-7185 (F.S.); +39-011-670-6862 (S.A.)

Abstract: α-amylases are ubiquitous enzymes belonging to the glycosyl hydrolase (GH13) family, whose members share a high degree of sequence identity, even between distant organisms. To understand the determinants of catalytic activity of α-amylases throughout evolution, and to investigate the use of homologous enzymes as a model for the human one, we compared human salivary α-amylase, *Aspergillus oryzae* α-amylase and pancreatic porcine α-amylase, using a combination of in vitro and in silico approaches. Enzyme sequences were aligned, and structures superposed, whereas kinetics were spectroscopically studied by using commercial synthetic substrates. These three enzymes show strikingly different activities, specifically mediated by different ions, despite relevant structural homology. Our study confirms that the function of α-amylases throughout evolution has considerably diverged, although key structural determinants, such as the catalytic triad and the calcium-binding pocket, have been retained. These functional differences need to be carefully considered when α-amylases, from different organisms, are used as a model for the human enzymes. In this frame, particular focus is needed for the setup of proper experimental conditions.

Keywords: α-amylases; *Aspergillus oryzae* α-amylase; human α-amylase; porcine α-amylase

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1. Introduction

α-amylases are ubiquitous enzymes, distributed in bacteria, fungi, animals and plants [1]. They belong to the glycosyl hydrolase (GH13) family, whose members share a high degree of sequence identity, even between distant organisms, such as mammals and bacteria [1–3]. These enzymes are essential for carbohydrate metabolism and their main function is to catalyze the hydrolysis of the α-1,4-glycosidic bond of polysaccharides, retaining the anomeric carbon configuration and forming oligosaccharides and smaller polysaccharides, which will be subsequently transformed into glucose units by the action of other hydrolytic enzymes [4]. In general, α-amylases are enzymes with broad substrate preference and product specificity [5].

From a structural point of view, all α-amylases present three different domains. The A domain assumes a TIM-barrel conformation and includes the active site of the enzyme enclosing the catalytic triad residues Asp197, Glu233, and Asp300 [6], acting as nucleophile, acid/base catalyst, and transition state stabilizer, respectively [5,7]. The B domain, on the other hand, is located between the third β-strand and the TIM-barrel helix and participates in maintaining protein stability and substrate binding [7]. Finally, the C domain is formed by eight β-strands and it is bound to the A domain (Figure 1) [8,9].

A general feature of all α-amylases is the presence of a calcium ion, bound to a strongly conserved site, located between domains A and B, and playing a major role in enzyme stability and activity [1,10,11]. Animal and some Gram-negative bacteria α-amylases also present a chloride ion, working as an allosteric activator [12–15]. Specifically, in mammalian

α-amylases the chloride ion opposes the action of the nearby arginine residues and allows the side-chain of Glu233 to be appropriately oriented and protonated at the physiological pH, which is required for the enzyme to perform its highest catalytic activity [1,16,17].

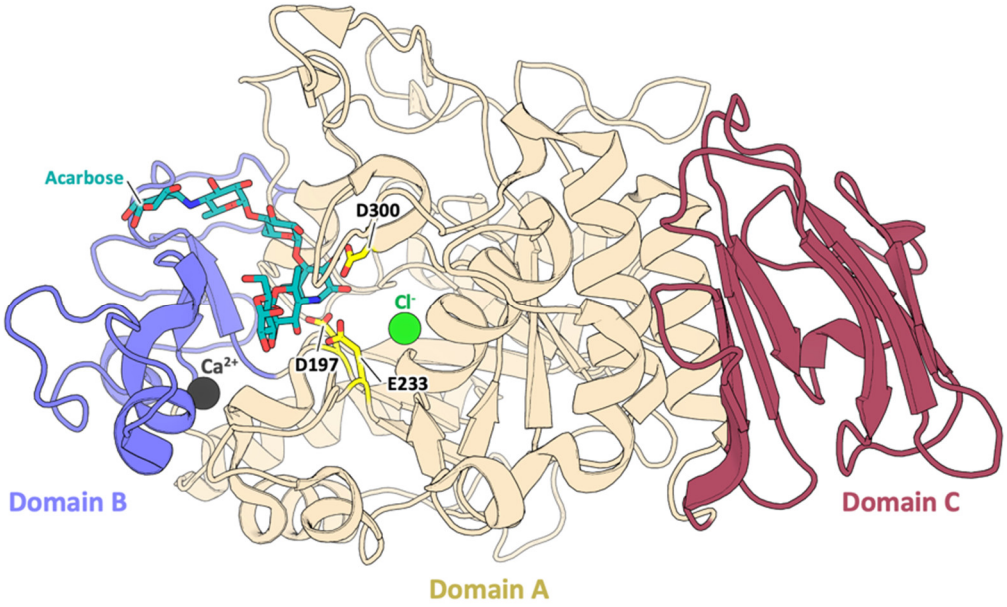

Figure 1. Folding and domains of α-amylases. Ions are represented as spheres, the catalytic triad and the substrate as sticks and the protein as cartoon (PDB ID: 1mfv; human α-amylase). The three domains (A, B, and C) are shown in different colors.

To understand the determinants of the catalytic activity of α-amylases among the species and investigate the use of homologous enzymes as a model for human enzymes, we compared human salivary α-amylase (HSA), *Aspergillus oryzae* α-amylase (AOA) and pancreatic porcine α-amylase (PPA), using a combination of complementary biochemical in vitro and in silico approaches. We showed that these enzymes present remarkably different activities mediated by different ions, despite their homology.

2. Materials and Methods

2.1. Modelling

The 3D structure of HSA, AOA and PPA, all complexed with acarbose, were retrieved from the Protein Data Bank (PDB ID 1mfv, 7taa and 1ose, respectively). The proteins were inspected and superposed with Pymol 2.5.2. (Schrödinger, Inc., New York, NY, USA).

The corresponding sequences were retrieved from UNIPROT and aligned with ClustalX. The UNIPROT codes are the following: HSA, P0DTE8; AOA, P0C1B3; PPA, P00690.

2.2. Chemicals and Enzymatic Activity

Chemicals were purchased from Sigma-Aldrich (Milan, Italy) as well as α-amylase from *A. oryzae*, *Homo sapiens* and *Sus scrofa*.

The enzymatic activity of α-amylases was determined by a colorimetric assay, based on the hydrolysis of the synthetic substrate p-nitrophenyl α-D-maltoheptaoside, blocked at the reducing end (Megazyme, Bray, Co., Wicklow, Ireland). This reaction product is subsequently hydrolyzed by α-glucosidases and amyloglucosidases, supplied together with the substrate, to produce the p-nitrophenolate anion, which can be quantified spectrophotometrically at 405 nm [18].

Each assay was run at least in duplicate at 37 °C on a 96-well multi-well plate, in which 200 µL reaction mixtures were added. The absorbance values were determined at 405 nm every 3 min for 1 h, by using the microplate reader EnSight (Perkin Elmer, Milan, Italy). Determinations were performed by using 10 mU of either enzyme in different buffer

systems: (i) 50 mM HEPES buffer, pH 7.0; (ii) 50 mM HEPES pH 7.0 buffer in the presence of 72 mM NaCl, and 1 mM $Ca(NO_3)_2$; (iii) 72 mM NaCl; (iv) 1 mM $Ca(NO_3)_2$; (v) 1 mM calcium lactate; (vi) 2 mM NH_4NO_3. The reaction was started adding 30 µL of the substrate solution to the reaction mixture, resulting in a final concentration of 0.3 mM.

3. Results

3.1. Sequence and Structural Comparison

The alignment of HSA with AOA returned 113 conserved residues, for a sequence identity of only 20%, and 148 similar residues. Differently, the alignment of HSA with PPA returned a quite high sequence identity of 86% (Figure S1). While we could assume that the structure of HSA and PPA is well maintained, we had to carefully investigate the structural similarities/differences between HSA and AOA, even if previous studies reported a conserved folding [19].

The structural superposition of HSA and PPA enzymes shows a very good alignment (RMSD equal to 0.3 Å; Figure 2).

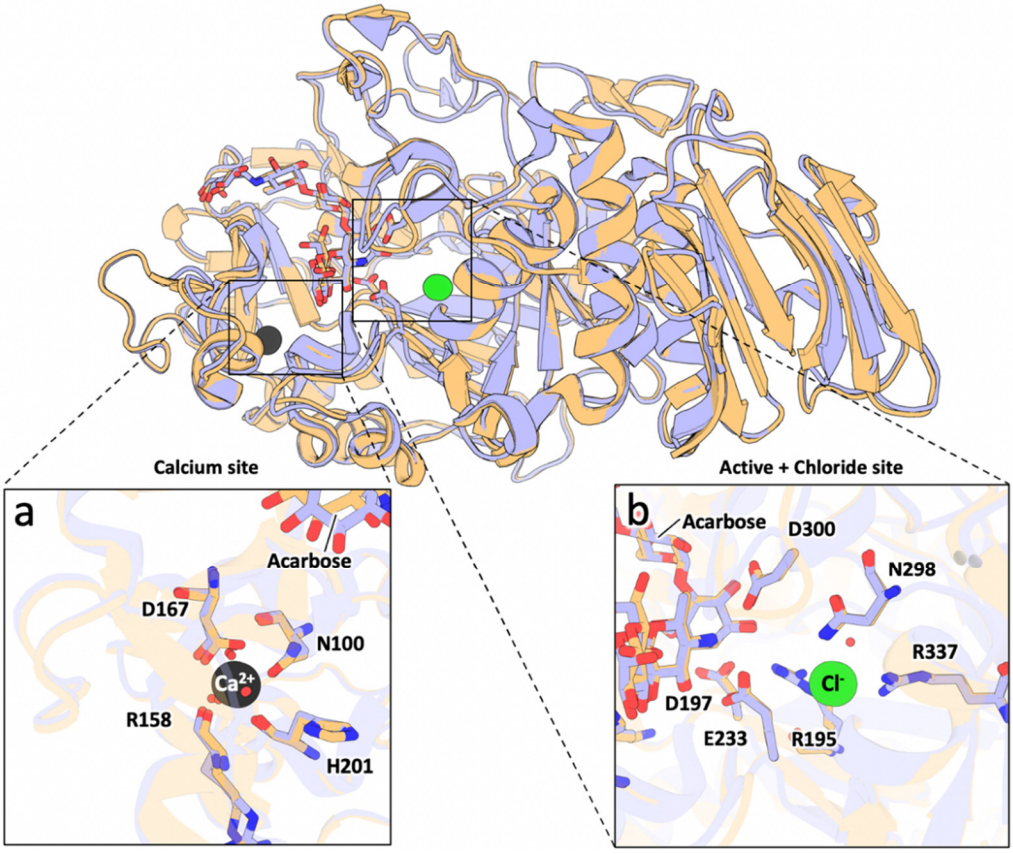

Figure 2. Structural superposition of HSA (lilac) and PPA (orange). Ions are represented as spheres, the catalytic triad and the substrate as sticks and the protein as cartoon (PDB ID: 1mfv and 1ose for HSA ad PPA, respectively). Coordinating water molecules are shown as red spheres. (**a**) Superposition of the calcium-binding site. (**b**) Superposition of the chloride-binding site.

The active site, located for all isoforms at the interface between domains A and B, is perfectly superposed, as is the cluster of residues coordinating the calcium and the chloride ions (Figure 2a,b, respectively). Further, the two ions occupy exactly the same position and, as previously mentioned, the coordinating residues are all conserved. This, along with the structural similarity, strongly suggests that the two enzymes work in a similar way, and both need ions to be completely active and structurally stable.

More significant discrepancies can be observed when comparing the structure, complexed with acarbose, of HSA and AOA (RMSD equal to 3.4 Å; Figure 3). While the

residues forming the catalytic triad are well superposed (Figure 3a; [19]); the upper part of the binding site belonging to the A domain appears quite shifted, possibly because of several gaps in the sequence alignment. Indeed, only a small fraction of active site residues is maintained in the same position, while many are not conserved or displaced. The cluster of residues coordinating the calcium ion is well maintained (Figure 3b), in accordance with previous studies [1]. On the contrary, the chloride ion is missing in AOA and important substitutions in the surrounding residues can be observed (Figure 3c; [17]).

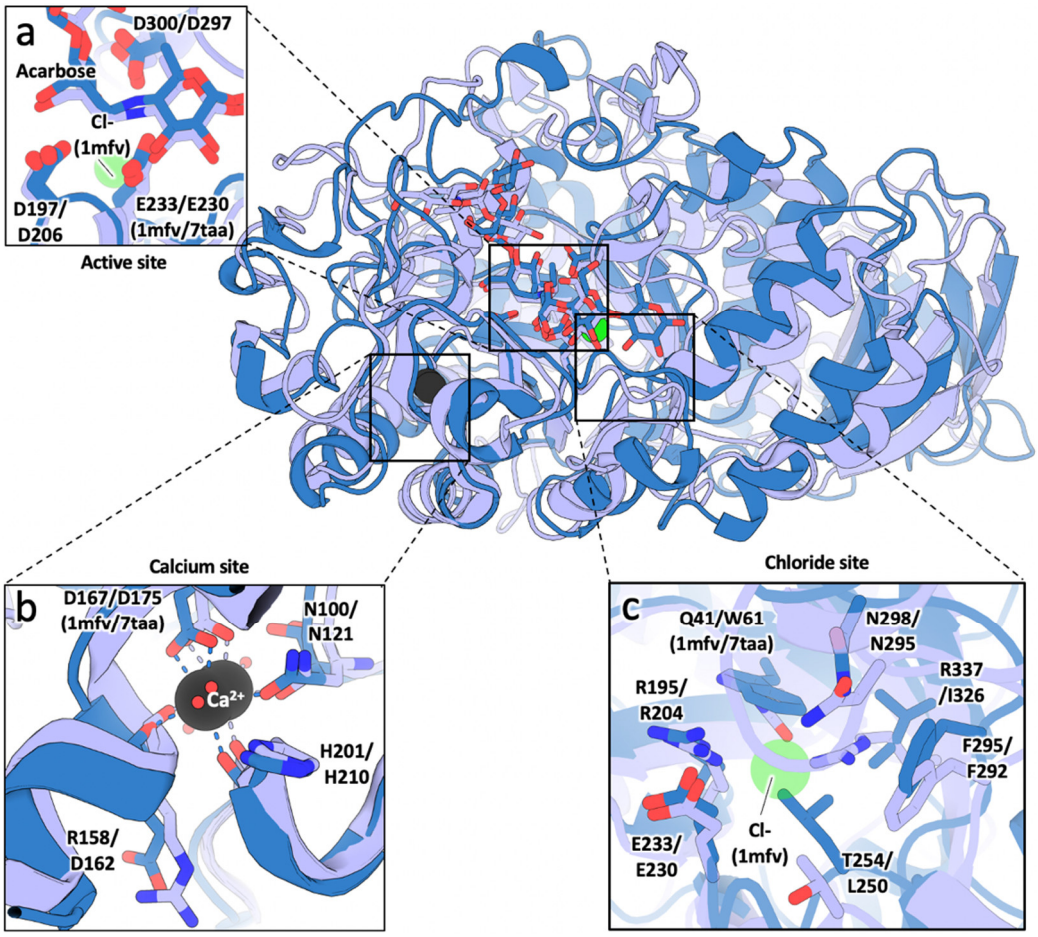

Figure 3. Structural superposition of HSA (lilac) and AOA (blue). Ions are represented as spheres, the catalytic triad and the substrate as sticks and the protein as cartoon (PDB ID: 1mfv and 7taa for HSA ad AOA, respectively). Coordinating water molecules are shown as red spheres. (**a**) Superposition of the catalytic triad (**b**) Superposition of the calcium-binding site. (**c**) Superposition of the chloride-binding site. Coordinating water molecules have been omitted for clarity.

3.2. Enzymatic Activity

Considering the structural similarities and differences among HSA, PPA and AOA, we decided to address their enzymatic activity and further evaluate the possibility of transferring information from one model to another.

The analysis of the enzymatic activity was performed via a spectrophotometric assay that uses, as a substrate, a blocked 4-nitrophenyl-α-maltoheptaoside, which, upon subsequent hydrolysis by the α-amylase and the α-glucosidases (supplied with the substrate), provides the free para-nitrophenolate anion, responsible for the absorbance increase at 405 nm. Tests were carried out in HEPES buffer at pH 7.0, which was preferred over phosphate buffer, generally used in the literature [20–22], since the phosphate ion could interact with the calcium ion, an essential element for the proper functioning of the enzyme.

Significant differences in the activity of the various α-amylases under examination are highlighted in Figure 4. Interestingly, in the absence of added ions, AOA (green squares)

is active, whereas HSA (black circles) and PPA (red triangles) show little activity. These findings suggest that AOA activity is not strictly dependent on the presence of ions, whereas HSA and PPA apparently need ions to be fully active. We, therefore, decided to further investigate the contribution of various ions to the activity of the different α-amylases.

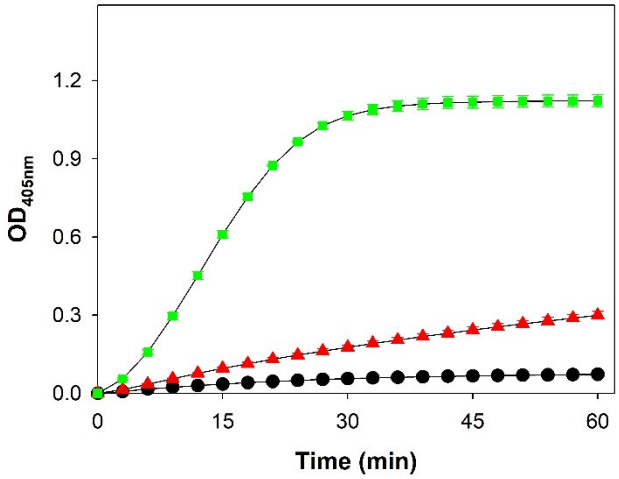

Figure 4. Enzymatic activity in the absence of added ions of HSA (black circles), PPA (red triangles) and AOA (green squares), measured as the absorbance increase in the various amylase solution at 405 nm.

Ion-deprived HSA presents very little enzymatic activity (green circles, Figure 5a), which significantly increases following the addition of specific ions. In particular, calcium (red circles) leads to a 4-fold increase, whereas chloride addition (blue circles) results in an impressive 12-fold rise. The simultaneous presence of both ions (cyan circles) leads to a further increase in HSA activity (about 16-fold with respect to the enzyme alone).

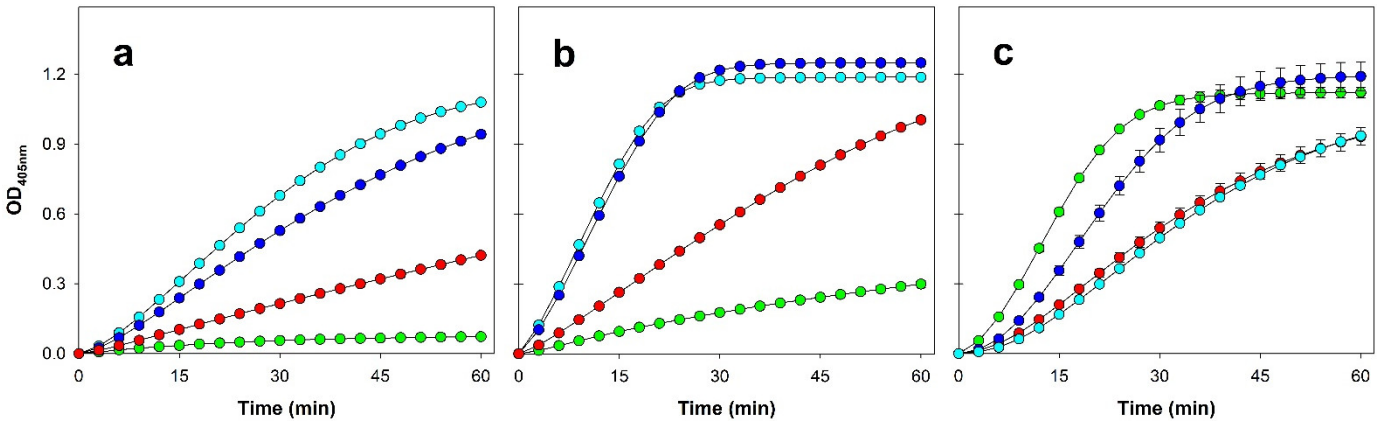

Figure 5. Effect of ions on HSA (**a**), PPA (**b**) and AOA (**c**) activity. The enzymatic activity was measured as the absorbance of the solution at 405 nm, in the absence of ions (green circles), in the presence of calcium (red circles), chloride (blue circles) or both ions (cyan circles).

PPA showed similar behavior with respect to HSA (Figure 5b). The addition of calcium (red circles) to the reaction mixture provided a relevant contribution to the enzymatic activity, as compared to the enzyme alone, resulting in a 3-fold activity increase, whereas chloride addition (blues circles) accounts for about an 8-fold increase in PPA activity. No further increase in enzyme activity was observed when both ions (cyan activity) were added together, clearly suggesting that the activity of PPA is mostly affected by chloride ions.

In the same conditions, AOA displays a quite different trend (Figure 5c). In particular, AOA shows the highest activity in the absence of ions (green circles), showing a radically

opposite behavior to HSA. Moreover, a dramatic inhibition of the enzyme activity can be observed when calcium (red circles) and chloride (blue circles) ions are added separately or in combination (cyan circles). Indeed, the addition of calcium nitrate in the reaction mixture as a source of calcium results in a 50% decrease in the activity, with respect to AOA alone. A comparable inhibition was recorded when the enzymatic activity was measured in the simultaneous presence of NaCl and $Ca(NO_3)_2$. The inhibitory effect of chloride alone was, on the contrary, less significant (about 14% inhibition).

As calcium was added as calcium nitrate, we further addressed the possible binding of nitrate in the chloride pocket and the consequent effect on the activity. We, thus, treated PPA with calcium nitrate and calcium lactate, assuming that this latter is such a bulky anion and should be unable to fit within the chloride pocket. The same experiment was already carried out on human amylases, showing that nitrate has an activating effect on the enzymatic kinetics, whereas larger anions (acetate, carbonate, and sulfate) showed no effect, most likely because they could not fit in the chloride binding pocket [21].

The effect of nitrate was also verified by treating the enzyme with ammonium nitrate, and assuming that the ammonium ion may be too bulky to fit within the amylase calcium-binding site. As previously shown, the enzyme has the lowest activity when tested in the absence of ions (green circles, Figure 6). On the contrary, the highest activity was measured in the presence of both calcium and chloride, provided as $Ca(NO_3)_2$ and NaCl, respectively (cyan circles; 7-fold increase). Interestingly, a significant activity increase of around 3-fold is observed upon addition of nitrate from $Ca(NO_3)_2$ and NH_4NO_3 (red and purple circles), thus, supporting the idea that nitrate can *per se* exert an activating effect. However, nitrate seems to be less effective with respect to chloride, which accounted for most of the activity increase when added as NaCl (Figure 5b). This evidence leads one to speculate that nitrate could be able to replace the chloride in the same binding pocket, even if not that efficiently, possibly because of the triangular and nearly planar structural feature that nitrate ion shares with chloride [13,21]. Indeed, nitrate binds strongly to α-amylase, but is a weak activator, possibly because of the delocalized charge with respect to that of chloride [23]. On the other hand, the anion behavior described above leads one to speculate that calcium has little effect on PPA activity, while it is fundamental in maintaining protein structural integrity. A less important effect has been measured when sodium lactate is added to the PPA enzyme (yellow circles), thus, confirming that lactate is too bulky and, therefore, stereochemically incompatible to fit in the chloride-binding site.

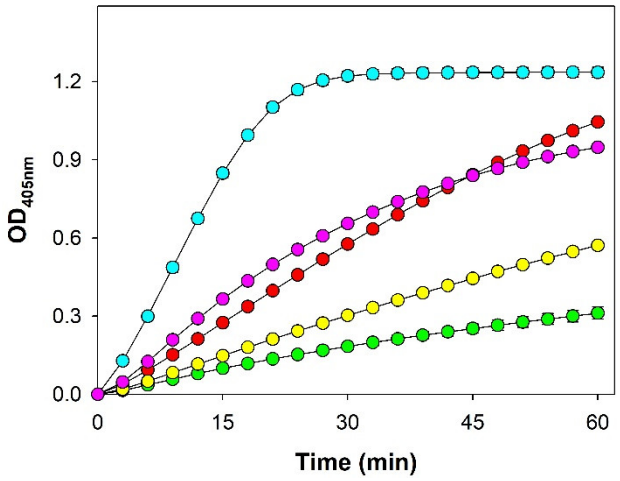

Figure 6. Effect of additional ions on PPA activity. The enzyme activity was measured as the absorption of the solution at 405 nm, in absence of ions (green circles), in presence of sodium lactate (yellow circles), NH_4NO_3 (purple circles), $Ca(NO_3)_2$ (red circles), $Ca(NO_3)_2$ and NaCl (cyan circles).

4. Discussion

The sequence and structural alignment of the proteins returned a quite conserved folding and a good similarity, in particular, at the binding-site level, where the catalytic triad and the calcium-binding site are maintained and perfectly aligned, in accordance with previous studies [10,24]. While PPA and HSA show an extremely high level of similarity, AOA presents relevant differences with respect to HSA, in particular, as for domain A and the chloride-binding site, in which two critical substitutions occur and no ion is bound.

The presence of calcium and chloride ions is essential for the optimal functioning of HSA. Although chloride has the predominant effect on enzyme activity, calcium is essential to achieve full functionality of the human enzyme. On the other hand, calcium and/or chloride ions are not required to ensure the full function of fungal amylase. In particular, calcium ions determine a quite marked inhibition of AOA activity, while chloride ions have no effect. The former finding is in agreement with the literature, where calcium ions at high concentrations have been shown to specifically inhibit the fungal protein [10]. These functional differences have to be carefully considered when using the fungal enzyme as a model for the human amylases, with particular attention on the setup of the experimental conditions. In addition, the use of the PPA as a cheaper substitute of the human enzyme is preferred, because these two enzymes share a similar activity behavior, also in the presence of chloride and calcium ions, clearly associated to a higher similarity in the sequence and in the structural architecture.

In fact, as with HSA, PPA is regulated by the presence of calcium and chloride ions. Their presence and, in particular, that of chloride, results in complete activation of the enzyme. The demonstration that calcium and chloride ions are essential for the activity of PPA has been shown by inserting the ions themselves into the reaction mixture, in the form of salts, and analyzing and excising the effects of the respective counterions.

5. Conclusions

Our results confirm the hypothesis that α-amylase function throughout evolution has considerably diverged, although key structural determinants, such as the catalytic triad and the calcium-binding pocket, have been retained. These functional differences have to be carefully considered when using the fungal enzyme as a model for human amylases, with particular attention to the setup of the experimental conditions.

Our investigation clearly suggests that PPA is a cheaper and better-suited substitute of the HSA, with respect to AOA, considering that they share similar activity behavior, specifically in the presence of chloride and/or calcium ions, and a higher structural similarity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr10040780/s1, Figure S1: Sequence alignment of HAS, PPA and AOA.

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Data Availability Statement: Data are available upon request to the corresponding authors.

Conflicts of Interest: The authors declare no conflict of interest.

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