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Bacillus Amyloliquefaciens Alpha-amylase Inhibition by Organic Solvents: A Study on Methanol, Ethanol and Propanol

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ABSTRACT

Alpha-amylase is widely used as an industrial enzyme, and a therapeutic target for which inhibitors are designed. Organic solvents are used to dissolve various compounds that would be studied as moderators of alpha-amylases, but they could themselves affect enzyme activity and stability. Methanol, ethanol and propanol are simple alcohols that may be commonly used to this end, and their effect has been investigated on the activity and stability of Bacillus amyloliquefaciens alpha amylase (BAA) enzyme. All three compounds were found to reversibly inhibit BAA, with methanol decreasing the binding affinity of substrate to BAA, and ethanol and propanol showing mixed type of inhibition. A docking experiment suggests the existence of a common binding site for the three alcohols. The proposed site is located near to one calcium binding site of the enzyme, a fact that correlates with the reduced thermal stability of BAA in presence of all three alcohols. In conclusion, the dose dependent inhibition of these solvents should be taken into account when studying the effect of moderators.

Keywords: Alpha-amylase, Bacillus amyloliquefaciens, Inhibition, Alcohols

INTRODUCTION

Organic solvents are used in the study of enzymatic reactions or in industrial processes, either as enzyme medium or to dissolve moderators whose effects are to be tested on an enzyme activity. These solvents may interfere in enzymatic reactions [1], and the ratio of water to organic solvents is an important factor when it comes to assess enzyme activity [1,2]. Most studies focus on enzyme-catalyzed reactions in presence of low water content, but such interferences may still be detected at high aqueous content with organic solvents of various concentrations [3].

Alpha-amylases (EC 3.2.1.1) hydrolyze alpha (1-4) endo-glycosidic bonds in starch and related compounds [4] and belong to the glycosyl hydrolases family 13 [5]. These enzymes are widely used in different processes including food, detergent, textile, and paper industry [6,7]. They have low similarity at sequence level but share a common central $(\alpha/\beta)_8$ barrel which contains the catalytic residues [8-10]. Thermostabilization [11] and inhibition [12] are two of the important research topics related to alpha-amylases. Inhibitors have been a matter of interest for years because they may have a role in controlling blood sugar levels and be effective in diseases such as diabetes and related metabolic disorders [13]. There are rare reports about the impact of organic solvents on these enzymes; in one study, ethanol has been reported to decrease activity in a halophilic alpha-amylase [14].

Some additives may also affect alpha-amylase activity. In a report by Yoon and Robyt, triton-X-100, polyethylene glycol (PEG) and poly vinyl alcohol (PVA) were tested at various concentrations on alpha-amylases from different sources and found to have activator and stabilizator roles [15]. Chloride ion was the first allosteric activator reported for this enzyme [16] after what more studies were done to

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clarify the details of this activation mechanism [17] and to find substituent compounds for chloride ion [18]. Recently, xanthine derivatives were observed to increase enzyme activity in a non-dose-dependent manner [19] and a chalcone derivative, neohesperidin dihydrochalcone, was reported as a non-essential activator of bacterial, fungal and mammalian alpha-amylase [20,21]. Due to the key role of this enzyme in the control of blood sugar level [13,20], most reports in literature concern the inhibitors of these enzymes. Proteinous and non-proteinous compounds acting by various mechanisms have been investigated in detail [13,22-25]. Flavonoids have been reported as promising inhibitors of this enzyme [26,27]and found to interact with active site residues [28]. Similarly, flavonoids precursor trans-chalcone is an inhibitor of mammalian amylase, and suggested to interact with the enzyme active site *via* pi-pi interaction [29]. Other studies have also reported the effect of plant extracts from which compounds other than flavonoids have been isolated and further studied [30,31]. Finally, natural compounds of microbial origin have been recently characterized asamylase inhibitors [32,33].

Propanol (C_3H_8O), ethanol (C_2H_6O) and methanol (CH_4O) are three primary alcohols that are used to solvate polar substances. To our knowledge, except for the pre-mentioned article on a halophilic amylase [14] , no report has been made on the effect of primary alcohol on alpha amylase. Since these solvents may be used during screening of alpha amylase moderators, this study was performed to investigate their potential effect on alpha-amylase activity and stability.

MATERIALS AND METHODS

Materials

Bacillus amyloliquefaciens alpha-amylase (BAA) and 3,5-dinitrobenzoic acid (DNS) were purchased from Sigma (St. Louis, MO, USA); 1-Propanol was obtained from Scharlau (Scharlab S.L, Barcelona, Spain). Soluble starch, maltose and other chemicals were obtained from Merck (Darmstadt, Germany).

Methods

Enzyme assay. Potential effects of the moderators were tested on BAA activity by applying Bernfeld method [34]. Assays were performed in phosphate buffer (a mixed solution of 100 mM K_2HPO_4 and 50 mM NaCl with pH 7.5 ± 0.01). BAA activity was 1.92 (IU/ml) in presence of 1.25% (w/v) starch as the substrate. Various concentrations of methanol, ethanol and propanol were incubated with the enzyme at 25 °C and after gently being stirred for 10 min, activity was detected by a Shimadzu UV-1800 spectrophotometer and activities were calculated by the UV-Probe software. The results were reported as apercentage of the control sample activity (activity in absence of the solvents). All tests were repeated at least three times to ensure reproducibility.

Kinetic analysis was performed based on Lineweaver-Burk (L-B) equation:

$$1/V1 = (Km'/Vmax') \times 1/[S] + 1/Vmax' \quad (1)$$

$K_{m'}$ stands for Michaelis constant in presence of effectors, V_1 for velocity in presence of the inhibitor and $V_{max'}$ for maximum velocity in presence of each mediator, [S] shows substrate concentration [35-37]. α and β values are defined, respectively, as the magnitude of interaction between substrate and moderator binding site and magnitude of catalytic constant. These calculations were obtained based on the liberalizing methods [37-39].

Heat-Stability experiments. In order to investigate the possible changes in the enzyme stability in presence of the solvents, based on previous experiments [21], a temperature of 50 °C was chosen. For each compound, the concentration that reduced enzyme activity to 50% of the control sample activity was picked up to perform the test. Sampling intervals were set on every 5 min and immediately after sampling, tubes were transferred to ice, incubated for 30 min, and tested for remaining activity. For each set of the tests, two controls (heat exposed and unexposed control) were applied. Activities were measured by before-mentioned methods and reported as a percentage of unheated control.

Docking experiments. Auto dock vina was used for docking experiment [40]. The 3BH4.pbd file for BAA (www.pdb.org/) was first processed by the use of MOE 2012.10 (Chemical Computing Group Inc., Montreal, Canada). After deletion of the additional molecules alpha-amylase and adjustment of the protonation state of the structure for neutral pH, BAA grid box of 68 × 62 × 78 points with a spacing 1.0 A° was defined while the grid box

center was put on x = 71.252, y = 28.59 and z = 28.365. Ligands were designed and minimized by the use of MOE 2012.10 for the docking process. Gasteiger charges were assigned to protein and ligand molecules. Exhaustiveness was set on 20 and a computer with eight processors was utilized for the computation. One hundred poses were generated for each solvent and images were prepared with MOE 2012.10.

Ki of each solvent was calculated based on binding affinity, by applying this equation:

$$\Delta G = RTln\text{Ki}$$

Where ΔG is representative for binding affinity free energy (kcal mol^{-1}), R the gas constant, 1.99 kcal $Kmol^{-1}$ and T is temperature in Kelvin, and Ki is the inhibition constant.

RESULTS AND DISCUSSION

Methanol, Ethanol and Propanol Inhibition of BAA

Bernfeld method [34] was applied to detect possible changes in BAA activity in presence of the three alcoholic solvents at various concentrations. Concentrations from 0.3-1545 µM of methanol were used. As shown in Fig. 1A, activity decreased alongside with an increase in concentration so that in presence of 1545 µM of methanol only 7 percent of the activity was retained. Such dose-dependent activity reduction was also observed for the two other solvents; ethanol (Fig. 1B) in a range of 0.038-1072 µM gradually decreased BAA activity down to 13%, while in a range of 0.03-819 µM of propanol, the activity reduced almost regularly so that finally only 7 percents was left in presence of 819 (µM) of the solvent (Fig. 1C). Therefore, all

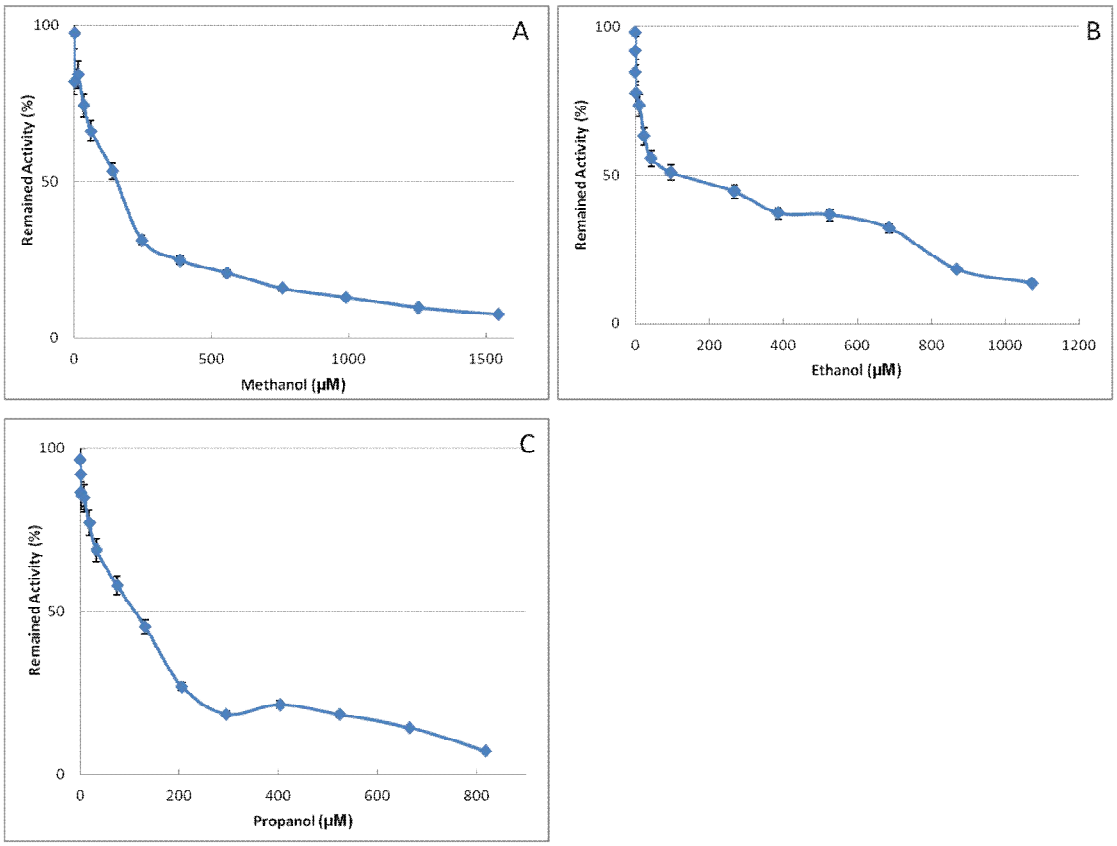

Fig. 1. Changes in activity (as a percentage of the sample control activity) (ordinate) are plotted against different concentrations of each solvent (abscissa); (A) methanol (B) ethanol and (C) propanol.

three solvents were considered as inhibitors of BAA; it is interesting to note that the more hydrophobic propanol was able to inhibit the enzyme at a lower concentration.

Reversible inhibition is easily reversed by dialysis or dilution [37] but in the irreversible type, where usually a covalent binding to the enzyme occurs, the effect is permanent. To investigate the inhibitory type, different concentrations of each solvent were plotted against the activity. In case of irreversible inhibition, a linear plot, and for reversible type, a non-linear plot is obtained [41]. Figure 2 shows the results of this test: none of the alcohols showed linear plot and all three inhibitors were found to be reversible inhibitors.

Kinetic Parameters of Inhibition

Kinetic parameters were obtained by L-B plot and replot. Double reciprocal plots for all three solvents are depicted in Fig. 3. First, ($1/V_{1'}$) and ($K_{m'}/V_{max'}$) *vs.* concentrations of each compound were plotted to find out if the inhibitions would be categorized as linear or non-linear type (Supplementary data 1) [38]. As observed in the Fig. 2, in all the three compound inhibition types were obtained as non-linear ones.

Methanol. Concentrations of 15, 139, 386, 759 and 1545 (µM) of methanol and six concentrations of starch ranging between 0.125-1.25% (w/v) were chosen to draw L-B plot (Fig. 3A). Since L-B secondary plots, slopes and intercepts *vs.* inhibitor concentration were non-linear (results not shown) differential method was applied to the rate equation to obtain kinetic parameters [38,42]. In this method, plotting 1/Δslope and 1/ΔY-intercept *vs.* reversed inhibitor concentration (1/[I]) results in straight lines and kinetic parameters are calculated according to the following rules: the Y-intercepts of the 1/Δslope and 1/ΔY-intercept

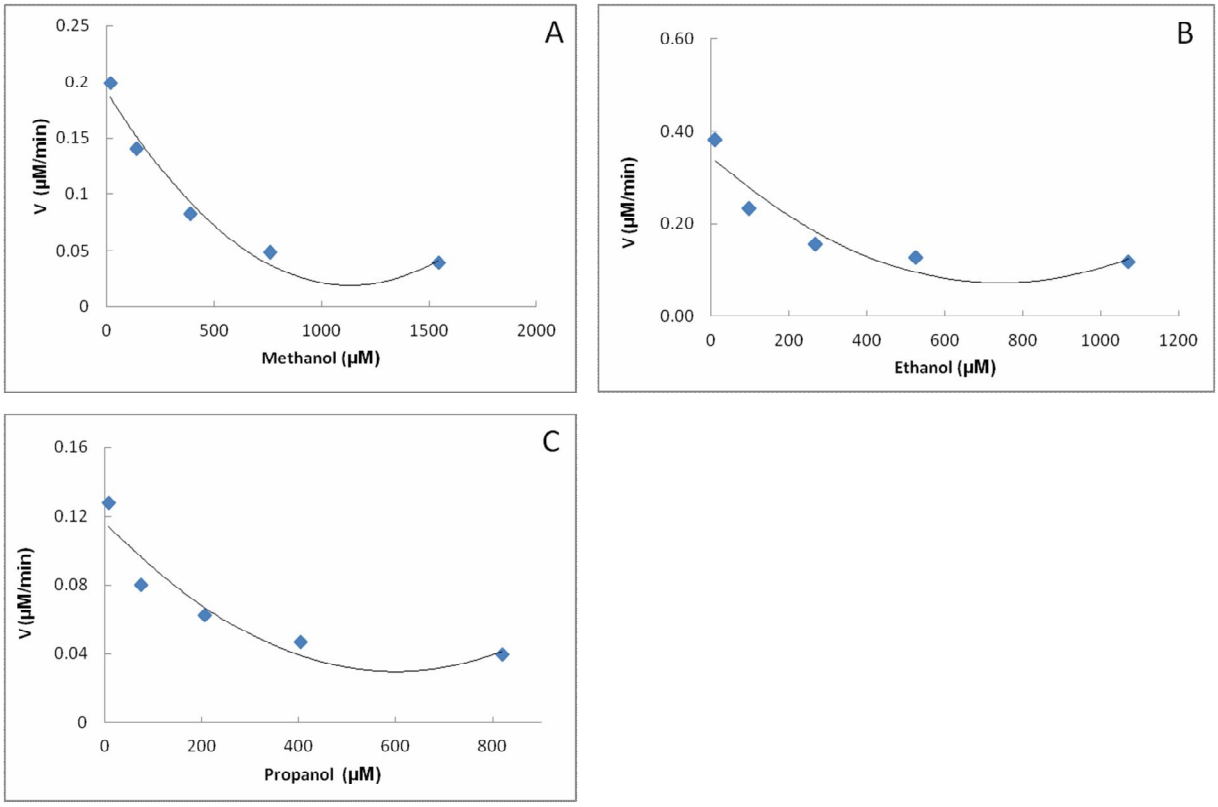

Fig. 2. Investigating the reversibility of the inhibition by each solvent. (A) Methanol, (B) ethanol and (C) propanol. Velocity in each concentration (Y-axis) is plotted against the same concentration of the alcohol (X-axis).

vs. (1/[I]), respectively equal to $1/V_{max}(\beta/(1-\beta))$ and $(V_{max}/K_m)(\beta/(\alpha-\beta))$ and the intersection of these two lines on abscissa is considered as $(-1/K_i)(\beta/\alpha)$. For methanol, α and β values were respectively 2.69 and 0.2 and K_i was 3.7 (μM) (Supplementary Data 2A). $\beta < 1 < \alpha < \infty$ is representative for hyperbolic non-competitive inhibition [38].

Ethanol. Five concentrations of ethanol (11, 97, 268, 526 and 1072 μM) were picked up for kinetic analysis in presence of four concentrations of the substrate: 0.125%, 0.375%, 1% and 1.25 % (w/v) (Fig. 3B). Kinetic parameters were calculated according the method described for methanol. Therefore α and β values were obtained as 0.00079 and 0.00026, respectively and K_i equaled 17 μM (Supplementar Data 2B). These data correspond to $0 < \beta < \alpha < 1$ which is hyperbolic noncompetitive inhibition [38].

Propanol. Concentrations of 8, 74, 205, 403 and 819 μM of propanol were tested in presence of different concentrations of starch at 0.125%, 0.2%, 1% and 1.25% (w/v) (Fig. 3C). Previously-mentioned kinetic analysis methods were applied; α and β values were calculated respectively as 0.022 and 0.0075. Ki was obtained to be 5.7 μM (Supplementary Data 2C). Again, the $0 < \beta < \alpha < 1$ pattern is representative for hyperbolic noncompetitive inhibition.

Concerning α and β, which are respectively representative for the affinity of substrate binding to the enzyme and an expression for catalytic constant, methanol

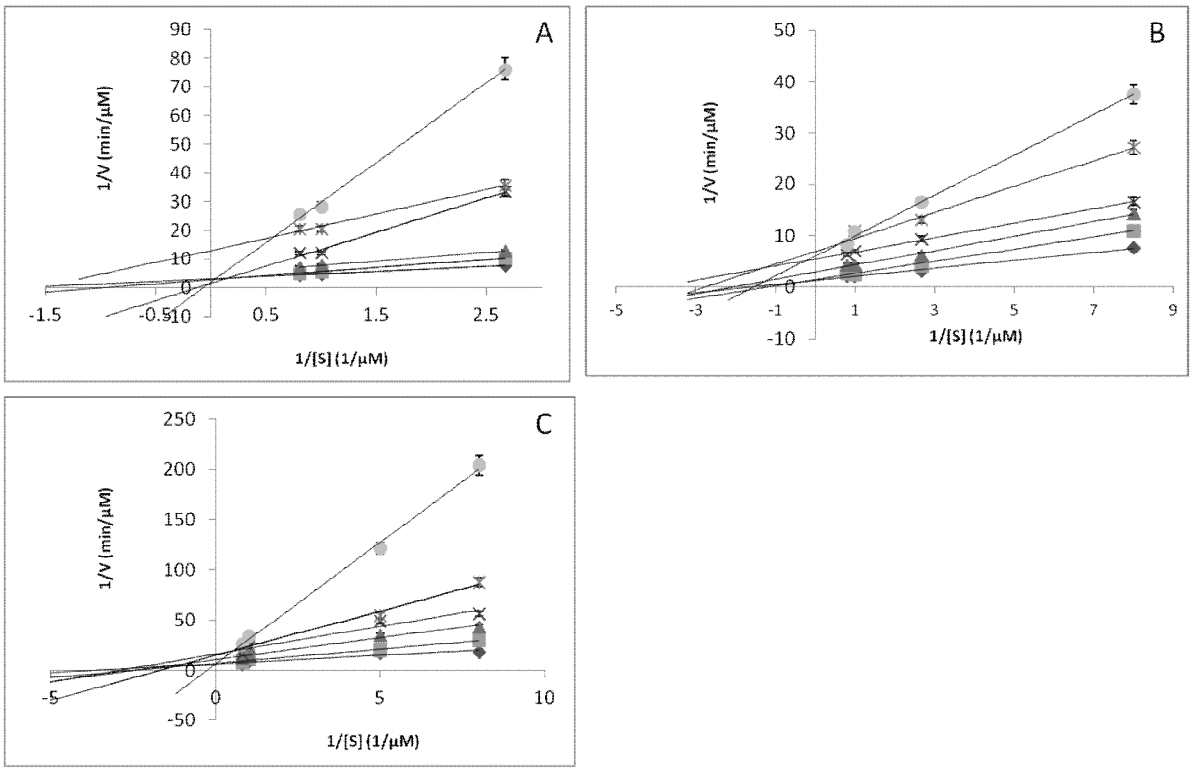

Fig. 3. Lineweaver-Burk Plots for (A) methanol, (B) ethanol and (C) propanol. In plot (A), symbols are used as follows: ♦ control sample, ■ 15 μM methanol, ▲ 139 μM of methanol, ✕ 386 μM of methanol, * 759 μM of methanol, and ● 1545 μM of methanol, in plot (B): ♦ control sample, ■ 11 μM of ethanol, ▲ 97 μM of ethanol, ✕ 268 μM of methanol, * 526 μM of ethanol, and ●1072 μM of ethanol and in plot (C): ♦ control sample, ■ 8 μM of propanol, ▲74 μM of propanol, ✕ 205 μM of propanol, * 403 μM of propanol, and ● 819 μM of propanol.

value which is $\alpha > 1$ shows negative cooperativity between substrate and inhibitor binding sites: binding of the substrate decreases inhibitor binding to the enzyme and *vice versa*. Therefore, it can be concluded that binding of the simplest alcohol, methanol, possibly reduces enzyme affinity to substrate [37]. On the other hand, deduced kinetic pattern of $\alpha < 1$ for ethanol and propanol represents cooperation between substrate and inhibitor binding and mixed type inhibition. Finally $\beta < 1$ for all three alcohols shows a decrease in catalytic constant which is expected for an inhibition process [37].

Docking Experiments: Finding the Binding Site of the Inhibitors

These experiments were performed to obtain more information about the putative binding sites and potential intermolecular interactions between the BAA protein as the receptor and the inhibitors as the ligands. The three alcohols were blind-docked on the receptor by the methods previously mentioned. Relative to the small size and presence of hydroxyl groups on the ligands, several binding sites could be found all over the receptor to be potential binding sites but most of them did not convey a rational explanation for an enzyme-inhibitor model, and had lower docking scores too. Consequently, those binding sites were selected which corresponded to high-scored ligand poses.

A binding pocket found as a common biding site for the highest-scored poses of all the three inhibitors is located between domains A and B and surrounded by four residues of the conserved calcium binding site G97-A109 and I217-H235 [43] (Fig. 4).

This pocket was formed by hydrophilic and less hydrophobic residues. Hydroxyl group of the methanol played a role as donor for F152 and as receptor for R145 to form two hydrogen bonds (Fig. 5). Hydrophilic interactions resulted into ethanol beng embraced by polar residues K105, R59, R145, Y59, Y61 (Fig. 6). Four of the highest scored poses of propanol were positioned in this pocket. Backbone carboxyl group of K60 made a hydrogen bond with the hydroxyl group of propanol (only highest scored pose is shown here) (Fig. 7).

Here too, increase in carbon content of the solvent results in better affinity to the enzyme and better settlement in the putative binding site because alongside with higher

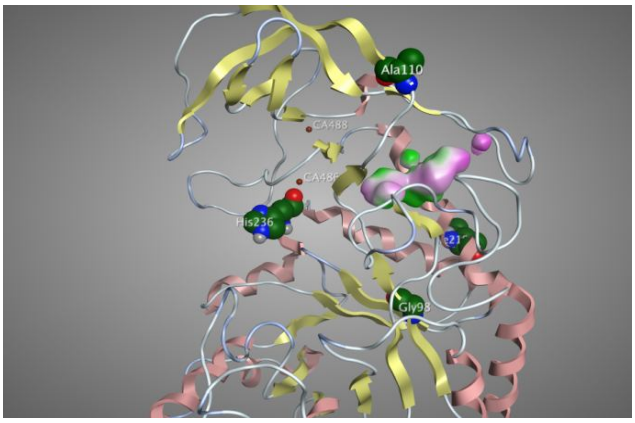

Fig. 4. Binding pocket of methanol, ethanol and propanol on BAA receptor. Ball shaped residues (in dark green) are the conserved calcium binding site residues. Ca^{2+} ions are also shown. The binding site of alcoholic solvents is located in an area between domain A and B and is embraced by calcium binding site residues.

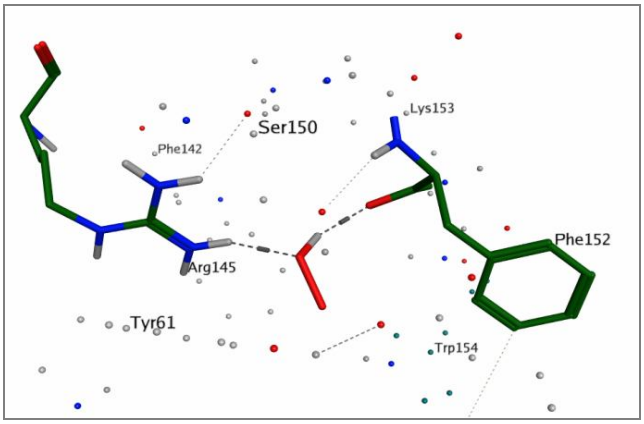

Fig. 5. Methanol binding site. Hydrogen bounds are formed between hydroxyl group of methanol and F152 and R145.

carbon content in the inhibitor structure, higher scores were obtained for docked poses (-2.1 kcal mol^{-1} for methanol, -2.8 kcal mol^{-1} for ethanol and -3.4 kcal mol^{-1} in the same location for propanol). Theoretical Ki values calculated for

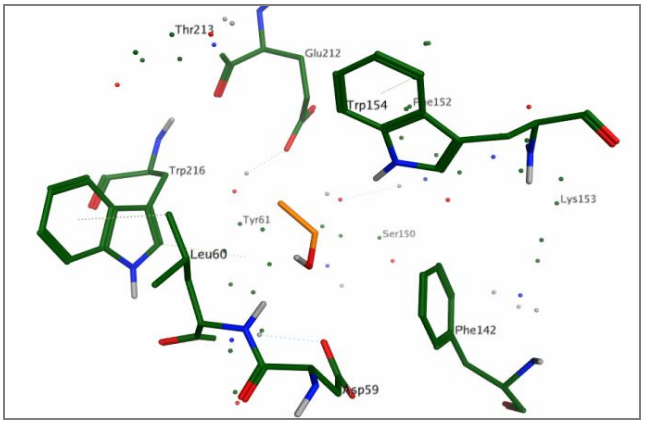

Fig. 6. Ethanol binding site. Ethanol was embraced in a pocket formed mostly of polar residues; K105, R59, R145, Y59, Y61.

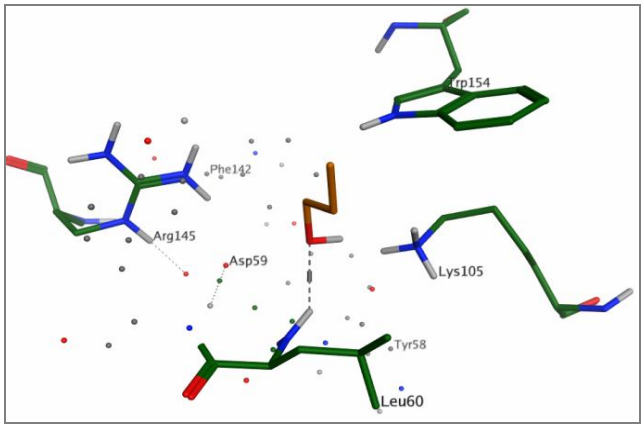

Fig. 7. Propanol binding site. A hydrogen bond formed with K60 is shown.

the binding affinities of methanol, ethanol and propanol were obtained equally at approximately 1 M. This result may be due to the slight difference found in binding energies of the ligands, which is related to the small size of the compounds and the multiplicity of putative binding sites. It is also interesting to note that excess calcium binding could decrease BAA activity spontaneously [44], as so, a potential role of the calcium binding region may be

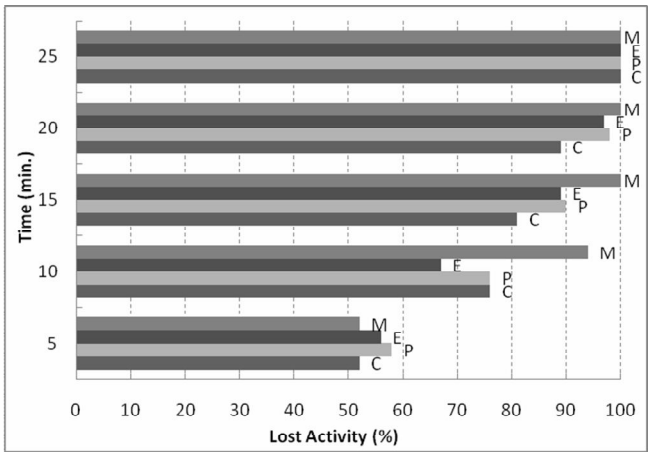

Fig. 8. Enzyme stability experiment results. Abscissa is representative for the lost activity (activity of heated samples relative to those for unheated control) and ordinate shows time in min. [C:control sample, M , E and P respectively stand for samples in presence of methanol, ethanol and propanol].

suggested in the inhibitory action of the compounds.

Enzyme Stability in Presence of the Inhibitors

The calcium binding region plays an essential role in BAA stability [45,46], and the predicted docked poses of the ligands are located close to some calcium binding residues. Enzyme stability tests were thus done to possibly provide further evidence about the putative binding site. As it was previously mentioned, experiments were performed at 50 °C and in 5 min intervals in presence of the inhibitors at concentrations that could reduce activity to 50% of the control sample activity. Results are depicted in Fig. 8 which shows that exposure to heat for 20 min resulted in no enzyme activity in presence of all solvents but control sample still retained 10 percent activity. After 25 min exposure to heat, the control sample was completely inactivated. As observed in Fig. 8, after 10 min, the rate of inactivation in control is lower than inhibitor samples. Figure 4 depicts the binding domain of the solvents which is close to one of the calcium binding regions in BAA [43]. Upon binding of calcium ion to the enzymes structure, the

content of α-helices changes and removal of calcium bound from the BAA structure can lead to conformational changes in BAA structure [45]. Binding of the solvents close to the calcium binding region may induce electrostatic changes in this area, and affect heat-stability of the enzyme in presence of these compounds.

Simple alcohols, especially methanol and propanol, are frequently used to solubilize compounds whose effects would be further tested on enzyme activity, and possible unwanted effects of such solvents on the activity could be a concern. Various reports exist about the interferences of the solvents in enzyme-catalyzed reaction process [47]. In some cases, they stabilize the enzyme structure or improve the catalysis process [48,49], and in others they may have negative impact on the process [50]. For alpha-amylases, there are few studies about the experimental microenvironment affecting the enzyme activity or stability. Tris buffer was found to be a competitive inhibitor of the *Bacillus licheniformis* alpha-amylase (BLA) [51]. In one case, ethanol was reported to inhibit a halophilic alpha-amylase [14], but we found no report about the effect of primary alcohols on bacterial alpha-amylase.

In conclusion, the current study shows the ability of primary alcohols to inhibit bacillus alpha-amylase in a noncompetitive way and may therefore interfere with the binding of other compounds to the enzyme; as so, these must be used with caution at higher concentrations. Further experiments are suggested to be performed to find out about such effects on the other members of family 13 hydrolases.

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REFERENCES

[1] M.N. Gupta, I. Roy, Eur. J. Biochem. 271 (2004) 2575.

[2] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81.

[3] D. Gangadharan, K.M. Nampoothiri, S. Sivaramakrishnan, A. Pandey, Appl. Biochem. Biotechnol. 158 (2009) 653.

[4] S. Ponnusamy, S. Zinjarde, S. Bhargava, P. Rajamohanan, A. RaviKumar, Food Chem. 135 (2012) 2638.

[5] B. Svensson, M. Tovborg Jensen, H. Mori, K.S. Bak-Jensen, B. Bonsager, P. K. Nielsen, B. Kramhoft, M. Prætorius-Ibba, J. Nohr, N. Juge, BIOLOGIA-BRATISLAVA- 57 (2002) 5.

[6] W. Aehle, O. Misset, Biotechnology Set, Second Ed. (1999) 189.

[7] R. Gupta, P. Gigras, H. Mohapatra, V.K. Goswami, B. Chauhan, Process Biochem. 38 (2003) 1599.

[8] J. Alikhajeh, K. Khajeh, B. Ranjbar, H. Naderi-Manesh, Y.H. Lin, E. Liu, H.H. Guan, Y.C. Hsieh, P. Chuankhayan, Y.C. Huang, J. Jeyaraman, M.Y. Liu, C.J. Chen, Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 66 (2010) 121.

[9] G.D. Brayer, Y. Luo, S.G. Withers, Protein Sci. 4 (1995) 1730.

[10] K.Y. Hwang, H.K. Song, C. Chang, J. Lee, S.Y. Lee, K.K. Kim, S. Choe, R.M. Sweet, S.W. Suh, Mol. Cells 7 (1997) 251.

[11] A.E. Habibi, K. Khajeh, H. Naderi-Manesh, B. Ranjbar, M. Nemat-Gorgani, J. Biotechnol. 123 (2006) 434.

[12] S.-H. Yoon, J.F. Robyt, Carbohyd. Res. 338 (2003) 1969.

[13] N. Mahmood, Comparative Clinical Pathology (2014) 1.

[14] T. Fukushima, T. Mizuki, A. Echigo, A. Inoue, R. Usami, Extremophiles 9 (2005) 85.

[15] S.H. Yoon, J.F. Robyt, Enzyme Microb. Tech. 37 (2005) 556.

[16] A. Levitzki, M.L. Steer, Eur. J. Biochem. 41 (1974) 171.

[17] N. Aghajari, G. Feller, C. Gerday, R. Haser, Protein Sci. 11 (2002) 1435.

[18] R. Maurus, A. Begum, L.K. Williams, J.R. Fredriksen, R. Zhang, S.G. Withers, G.D. Brayer, Biochemistry 47 (2008) 3332.

[19] E. Kashani-Amin, P. Yaghmaei, B. Larijani, A. Ebrahim-Habibi, Obesity Research & Clinical Practice.

[20] E. Kashani-Amin, B. Larijani, A. Ebrahim-Habibi,

FEBS lett. 587 (2013) 652.

[21] E. Kashani-Amin, A. Ebrahim-Habibi, B. Larijani, A.A. Moosavi-Movahedi, J. Mol. Recognit. (2015).

[22] M.J. Kim, S.B. Lee, H.S. Lee, S.Y. Lee, J.S. Baek, D. Kim, T.W. Moon, J.F. Robyt, K.H. Park, Archives of biochemistry and biophysics 371 (1999) 277.

[23] S.B. Larson, J.S. Day, A. McPherson, Biochemistry 49 (2010) 3101.

[24] T. Honda, Y. Kaneno-Urasaki, T. Ito, T. Kimura, N. Matsushima, H. Okabe, A. Yamasaki, T. Izumi, Drug Metab. Dispos 42 (2014) 326.

[25] S. Ponnusamy, S. Zinjarde, S. Bhargava, P.R. Rajamohanan, A. Ravikumar, Food Chem. 135 (2012) 2638.

[26] E. Lo Piparo, H. Scheib, N. Frei, G. Williamson, M. Grigorov, C.J. Chou, J. Med. Chem. 51 (2008) 3555.

[27] B. Nickavar, L. Abolhasani, Iran. J. Pharm. Res. 12 (2013) 57.

[28] L.K. Williams, C. Li. S.G. Withers, G.D. Brayer, J. Med. Chem. 55 10177.

[29] M. Najafian, A. Ebrahim-Habibi, N. Hezareh, P. Yaghmaei, K. Parivar, B. Larijani, Mol. Biol. Rep. 38 (2011) 1617.

[30] B. Nickavar, G. Amin, C. Zeitschrift fur Naturforschung, J. Biosci. 65 (2010) 567.

[31] R.M. Perez-Gutierrez, M. Damian-Guzman, Biol. Pharm. Bull. 35 (2012) 1516.

[32] P. Meng, Y. Guo, Q. Zhang, J. Hou, F. Bai, P. Geng, G. Bai, Carbohyd. Res. 346 (2011) 1898.

[33] A. Sokocevic, S. Han, J.W. Engels, Biochim. Biophys. Acta 1814 (2011) 1383.

[34] P. Bernfeld, Methods in Enzymology I (1955) 149.

[35] M. Dixon, E.C. Webb, Enzymes. Academic Press, New York 332 (1979).

[36] I.H. Segel, Enzyme Kinetics, Wiley, New York, 1993.

[37] A. Saboury, J. Iran. Chem. Soc. (JICS) 6 (2009) 219.

[38] V. Leskovac, Comprehensive Enzyme Kinetics, Springer, 2003.

[39] A. Saboury, M. Alijanianzadeh, J. Chin. Chem. Soc. 55 (2008) 937.

[40] O. Trott, A.J. Olson, J. Comput. Chem. 31 (2010) 455.

[41] S.R. Mikkelsen, E. Cortón, Bioanalytical Chemistry, John Wiley & Sons, Inc., 2004.

[42] W.W. Cleland, Adv. Enzymol Relat Areas Mol. Biol. 29 (1967) 1.

[43] A. Tanaka, E. Hoshino, J. Biosci. Bioeng. 96 (2003) 262.

[44] A.A. Saboury, Biologia, Bratislava 57 (2002) 221.

[45] H.S. Oh, K.H. Kim, S.W. Suh, M.-U. Choi, Korean Biochem. J. 24 (1991) 158.

[46] A. Tanaka, E. Hoshino, Biochem. J. 364 (2002) 635.

[47] M. Gupta, EJB Reviews, Springer Berlin Heidelberg, 1993.

[48] A.D. Blackwood, C. Bucke, Enzyme Microb. Tech. 27 (2000) 704.

[49] H. Ogino, H. Ishikawa, J. Biosci. Bioeng. 91 (2001) 109.

[50] V. Uchaipichat, P.I. Mackenzie, X.-H. Guo, D. Gardner-Stephen, A. Galetin, J. B. Houston, J.O. Miners, Drug Metab. Dispos. 32 (2004) 413.

[51] Z. Ghalanbor, N. Ghaemi, S.-A. Marashi, M. Amanlou, M. Habibi-Rezaei, K. Khajeh, B. Ranjbar, Protein Peptide lett. 15 (2008) 212.