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Review

Thermophilic archaeal amylolytic enzymes

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Abstract

The discovery of thermophilic Archaea was one of the major scientific events of the last years, especially because their enzymes are of great interest. Effectively, these later concern numerous biotechnological applications such as the enzymatic starch-hydrolysis in glucose production. In this review, we examine the different thermophilic archaeal amylolytic enzymes already characterized, with particular attention to α-amylases, the most studied enzyme of the glycoside-hydrolase family. The ecological importance of these enzymes, as well as their biochemical properties, their structure, and their evolutionary relationships are described.

Keywords: α-Amylase; α-Glucosidase; Pullulanase; Amylolytic enzyme; Archaea; Thermophilicity

1. Introduction

Industrial glucose production from starch is a two-step process involving a number of amylolytic enzymes (α-amylases, α-glucosidases, pullulanases) each having a different pattern of action on starch (Fig. 1). The first step (i.e. liquefaction) converts a concentrated starch suspension (30–40%) into a solution of soluble dextrins having different degrees of polymerization. During the second step (i.e. saccharification) these dextrins are hydrolyzed to glucose.

Because solubilized starch is a better substrate for amylases than insoluble starch, and because starch gelatinization only takes place at high temperatures (temperatures $>$ 70°C according the starch origin), starch enzymatic hydrolysis is now performed at elevated temperatures (90–110°C), by using thermophilic amylases. Such enzymes can be derived from mesophilic enzymes by chemical modifications or mutagenesis [1–7], but natural thermophilic amylases exist. *Bacillus stearothermophilus* and *B. licheniformis* α-amylases are well characterized, and are heavily used in the starch processing industry. Thermophilic amylases are also found in thermophilic and hyperthermophilic Archaea (Table 1) [8–10].

Enzymes used today in starch processing have varying temperature and pH requirements according their thermostability and physicochemical properties (Fig. 1). Performing starch liquefaction and saccharification in similar conditions of pH and temperature would decrease the cost of glucose-production. The development of saccharifying amylolytic enzymes that are active at high temperatures (i.e. ~90°C) would directly benefit the starch-processing industries.

Because numerous hyperthermophilic Archaea degrade starch, a great deal of effort has been spent recently in characterizing their amylolytic enzymes. These enzymes are interesting not only because of their biotechnological potentialities, but also because they can be used as models to study mechanisms of hyperthermophilicity. This review gives an overview of thermophilic archaeal amylolytic enzymes; eubacterial enzymes (i.e. from *Thermotoga* and *Thermus*) are not covered. We will particularly focus on α-amylases, which are the best studied of the amylolytic enzymes.

2. Archaeal α-amylases

α-Amylases (1,4-α-D-glucan, 4-glucanohydrolase; EC 3.2.1.1) hydrolyze α-1,4 linkages in starch. Some of them can also attack the α-1,6 bonds but with a lower efficiency.

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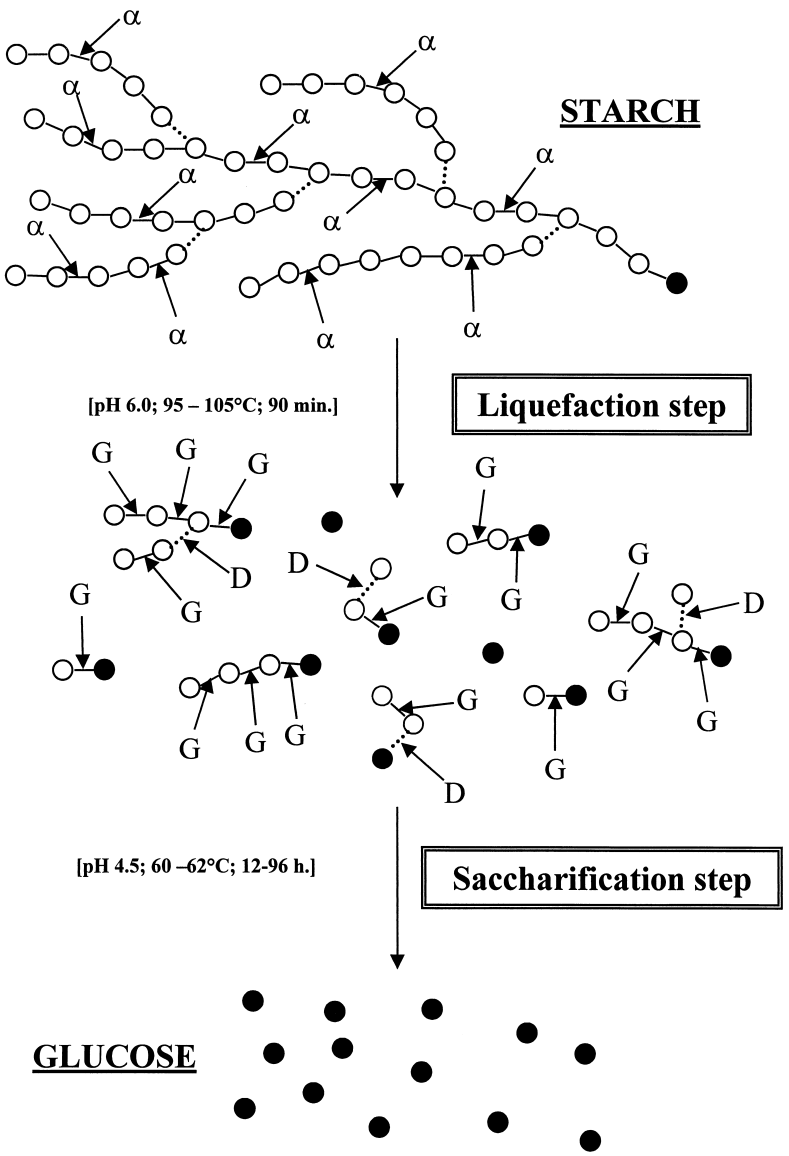

Fig. 1. Industrial enzymatic hydrolysis of starch into glucose and pattern of action of amylolytic enzymes. _____: α-1,4 glucosidic linkages; . . . . . : α-1,6 glucosidic linkages; A: α-amylase; G: glucosidase; D: debranching enzyme (pullulanase); O: non-reducing α-D-glucose residue; ●: reducing α- D-glucose residue.

The action of these enzymes on starch leads to a rapid decrease of the starch solution viscosity. α-Amylases are classified in two categories depending on the extent to which they hydrolyze starch. Liquefying α-amylases hydrolyze 30% to 40% of starch and saccharifying α-amylases hydrolyze 50% to 60% [11]. Most α-amylases adopt a common $(\alpha/\beta)_8$-barrel structure (Fig. 2) and belong to glycoside-hydrolases family 13 [12]. Thermophilic α-amylases are used in starch liquefaction, producing dextrins and α-limit-dextrins (branched dextrins) with different degrees of polymerization (Fig. 1).

Numerous hyperthermophilic Archaea (Table 1), especially deep-sea Thermococcale and *Sulfolobus* species contain α-amylases [8,9,13,14]. The genes encoding some of these enzymes have already been cloned and sequenced. This is the case for the *Pyrococcus furiosus* intracellular α-amylase [15] and for the extracellular α-amylases from *P. furiosus* [16–18], *Pyrococcus* sp. KOD1 [19], *Thermococcus profundus* [20], *Thermococcus hydrothermalis* [21,22], *Thermococcus* sp. Rt-3 [23], *Sulfolobus acidocaldarius*, and *Sulfolobus solfataricus* [24,25]. The gene encoding the α-amylase from the haloalcalophilic archaeum *Natronococcus amylolyticus* has also been cloned [26].

α-Amylases from Thermococcales are typical α-amylases (EC 3.2.1.1). In contrast, despite belonging to glycoside-hydrolases family 13, Sulfolobale and *N. amylolyticus* α-amylases differ from the other family 13 α-amylases by their activities. Sulfolobale α-amylases have glycosyl-trehalose-hydrolyzing activity and the *N. amylolyticus* enzyme has a maltotriose-forming activity [24–26].

Table 1
Thermophilic archaeal amylolytic enzymes and some of their characteristics

Archaea	Habitat	Enzymes	Optimal temperature of activity (°C)[a]	Optimal pH of activity[a]	Localisation of the enzyme	References
Desulfurococcus mucosus	hot solfataric spring	α-amylase	100	5.5		38
		pullulanase	100	5.0		38
Pyrococcus furiosus	marine solfatare	α-amylase	100	6.5–7.5	intracellular	30
		α-amylase	100	5.0	extracellular	40
		α-glucosidase	105–115	5.0–6.0	intracellular	44
		pullulanase	98	5.5	extracellular	52
Pyrococcus woesei	marine solfatare	α-amylase	100	5.5	extracellular	29
		α-glucosidase	110	5.0–5.5	intracellular	45
		pullulanase	100	6.0	cell-associated	55
Staphylothermus marinus	deep-sea hydrothermal vent	α-amylase	100	5.0		38
Sulfolobus shibatae	geothermal mud hole	α-glucosidase	85	5.5	intracellular	46
Sulfolobus solfataricus	volcanic hot spring	α-amylase	nd	nd	extracellular	28
		α-glucosidase	105	4.5	intracellular	47
Thermococcus aggregans	deep-sea hydrothermal vent	α-amylase	100	5.5		38
		pullulanase	100	6.5		38
Thermococcus celer	marine solfatare	α-amylase	90	5.5		38, 54
		pullulanase	90	5.5		38, 54
Thermococcus guaymagensis	deep-sea hydrothermal vent	α-amylase	100	6.5		38
		pullulanase	100	6.5		38
Thermococcus hydrothermalis	deep-sea hydrothermal vent	α-amylase	75–85	5.0–5.5	extracellular	21
		α-glucosidase	110	5.0–5.5	intracellular	49
		pullulanase	95	5.5	extracellular	56
Thermococcus litoralis	marine solfatare	pullulanase	98	5.5	extracellular	52
Thermococcus profundus	deep-sea hydrothermal vent	α-amylase	80	5.5–6.0	extracellular	37
Thermococcus zilligii	hot pool	α-glucosidase	75	7.0	extracellular	48
Thermococcus strain ST489	deep-sea hydrothermal vent	pullulanase	80–95	nd	extracellular	57

[a]nd, not determined by the authors.

3. Ecological importance of α-amylases from deep-sea Archaea

Because starch is rare in deep-sea hydrothermal vents, the presence of α-amylases in microorganisms living in these ecological niches is rather surprising. From a trophic point of view, deep-sea communities depend on the material sinking from the euphotic area (lightened upper layers of the ocean). However, more than 90% of the organic matter produced at the ocean surface is degraded in shallow zones, and almost no organic matter actually reaches the depth of 1200 m [27]. Instead, the actual substrate for these enzymes might be the glycogen produced by the animals and/or by other Archaea living in these deep-sea environments. By degrading the glycogen found in dead animals, Archaea could act as necrophages. Moreover, the fact that α-amylases are constitutively synthesized by some hyperthermophilic Archaea [28,29] suggests that these enzymes have an important role in these organisms' metabolism. This observation also supports the idea that their substrate is constantly available in deep-sea environments. Most α-amylases are extracellular enzymes (Table 1). In addition to extracellular α-amylases, *P. furiosus* contains an intracellular α-amylase [30]. The existence of this enzyme suggests that *P. furiosus* accumulates a polysaccharide reserve material. The fact that the *P. furiosus* intracellular α-amylase has a transglycosylating and/or synthesizing activity [30] indicates that this enzyme could be implied in the accumulation of this reserve material. Several works have indeed shown the existence of a polysaccharide reserve material in methanogenic Archaea belonging to the genus *Methanococcus*, *Methanotrix*, and *Methanosarcina* [31–34], as well as in thermoacidophilic Archaea belonging to the genus *Desulfurococcus*, *Sulfolobus*, *Thermococcus*, and *Thermoproteus* [35]. Because of the 30- to 40-kDa exclusion-size of the archaeal "S-layer" [36] prohibiting starch or glycogen from passively penetrating the cell, Archaea are likely to synthesize their polysaccharide reserve material.

4. Properties of thermophilic archaeal α-amylases

For the most part, thermophilic archaeal α-amylases do not differ from their mesophilic counterparts in molecular weight and amino acid composition. With the exception of *P. furiosus* intracellular α-amylase, which is a homodimer

Fig. 2. Barley α-amylase three-dimensional structure [65]. The structure consists of three domains: A, a parallel $(\alpha/\beta)_8$-barrel; B, an irregular fold stabilized by three Ca^{2+} ions (asterix); and C, a 5-stranded anti-parallel β-sheet. The barley α-amylase structure was chosen to illustrate the most probable structure of archaeal α-amylases based on the sequence similarities and evolutionary relationships found between the plant and archaeal α-amylases (see Chap. 7) [21,61].

with a subunit molecular weight of 66 kDa [30], all thermophilic archaeal α-amylases are monomeric enzymes with a molecular weight between 42 and 68 kDa [18,19,21,29, 37]. When expressed in the mesophilic Eubacteria *Escherichia coli* or *Bacillus subtilis*, the recombinant archaeal α-amylases retained all the biochemical properties of the native enzymes.

4.1. Effects of pH and temperature on activity

Thermococcale α-amylases are generally active in a broad pH range (pH 3.5–9) (Table 2). Their optimal pH for activity (pHopt) is typically between pH 5 and 6, except for the *Pyrococcus* sp. KOD1 (pHopt 6.5) [19] and *Thermococcus aggregans* (pHopt 6.5) [38] enzymes and for the *P. furiosus* intracellular α-amylase (pHopt 6.5–7.5) [30].

In general, *Thermococcus* α-amylases are less thermophilic than the *Pyrococcus* ones. *Thermococcus* α-amylases are optimally active at temperatures close to 80°C, whereas *Pyrococcus* enzymes are optimally active around 100°C (Table 2). These differences reflect the differences in optimal growth temperatures of these bacteria. Exceptions to this correlation between optimal growth temperature of a strain and optimal temperature of activity of its α-amylase(s) exist, however: with a Topt of 90°C, the α-amylase from the mesophile *B. licheniformis* is a good example. Thermococcale α-amylases are generally active in a broad temperature range, between 40°C and 140°C (Table 2).

Table 2
Physicochemical properties of the thermophilic archaeal α-amylases

Archaea	pHmin*	pHopt*	pHmax*	Tmin* (°C)	Topt* (°C)	Tmax* (°C)	Activating ions	References
D. mucosus	nd	5.5	9.0	60	100	110	nd	38
P. furiosus								
Extracellular form	3.5	5.0	8.0	40	100	140	none	40
Extracellular form	nd	5.6	nd	50	>100	nd	nd	39
Intracellular form	nd	6.5–7.5	9.0	40	100	120	none	30
P. woesei	3.5	5.5	>7.5	40	100	130	CA^{2+}, Co^{2+}, Mo^{2+}	29
P. sp. KOD1	4.0	6.5	>8.0	nd	90	110	none	18, 19
T. aggregans	nd	6.5	8.0	60	100	110	nd	38
T. celer	nd	6.0	8.0	60	90	110	nd	38, 54
T. guaymagensis	nd	5.5	8.0	60	100	120	nd	38
T. hydrothermalis	3.5	5.0–5.5	8.0	40	75–85	120	Ca^{2+}	21
T. profundus	4.0	5.5–6.0	8.5	40	80	100	Ca^{2+}, Cs^{2+}, Mg^{2+}	37

* nd, not determined by the authors.

Table 3
Effect of the metallic ions on the activity of the Thermococcales α-amylases. Values in brackets indicate the remaining activity in presence of the metallic ions.

Source	Activation	Poor inhibition	Strong inhibition	No effect	References
P. furiosus (extra-cellular enzyme)		2 mM Co^{2+} 2 mM Fe^{2+} 2 mM Ni^{2+}	2 mM Cr^{3+} 2 mM Cu^{2+} 2 mM Zn^{2+}	2 mM Ca^{2+} 2 mM Mg^{2+} 2 mM Mo^{2+}	40
P. furiosus (intra-cellular enzyme)		Co^{2+}, Cr^{2+}, Cu^{2+}, Fe^{2+} Mg^{2+}, Mn^{2+}			30
P. woesei	1 mM Ca^{2+} (120%) 1 mM Co^{2+} (120%) 3 mM Mo^{2+} (120%)	3 mM Ca^{2+} (85%) 2 mM Mg^{2+} (85%) 3 mM Co^{2+} (80%) 3 mM Ni^{2+} (50%)	3 mM Fe^{2+} (15%) 3 mM Cr^{2+} (1%) 3 mM Cu^{2+} (0%) 2 mM Zn^{2+} (0%)		29
Pyrococcus sp. KOD1		1 mM Co^{2+} (48%) 1 mM Mn^{2+} (45%)	1 mM Cu^{2+} (0%) 1 mM Zn^{2+} (0%)	1 mM Sr^{2+} (97%) 1 mM Ba^{2+} (96%) 1 mM Mg^{2+} (93%) 1 mM Ca^{2+} (92%)	19
T. hydrothermalis	0.1 mM Ca^{2+} (140%)	5 mM Ni^{2+} (83%) 5 mM Mg^{2+} (55%)	1 mM Mn^{2+} (2%)	5 mM Cu^{2+} (103%)	21
P. profundus	1 mM Ca^{2+} (115%) 1 mM Cs^{2+} (114%) 1 mM Mg^{2+} (113%)	1 mM Mn^{2+} (82%) 1 mM Ni^{2+} (80%) 1 mM Cd^{2+} (69%) 1 mM Ba^{2+} (67%) 1 mM Zn^{2+} (66%) 1 mM Cu^{2+} (44%)	1 mM Hg^{2+} (0%)	1 mM Co^{2+} (106%) 1 mM Fe^{2+} (102%) 1 mM Sr^{2+} (101%)	37

Optimally active between 100°C and 110°C [39,40], *P. furiosus* (strain DSM3638) extracellular α-amylase is the most thermophilic one.

4.2. Effect of metals on activity

α-Amylases are Ca^{2+}-dependent enzymes. Ca^{2+} is typically needed for activity and stability. Although Ca^{2+} has often been reported as an activator of archaeal α-amylases, a majority of these enzymes are active even in the absence of Ca^{2+}. To our knowledge, only two α-amylases do not require Ca^{2+}: archaeal *P. furiosus* and eubacterial *Thermus* sp. extracellular α-amylases [40,41]. Nevertheless, in these latter enzymes, it is possible that calcium can be bound so tightly to the enzyme that ethylenediaminetetraacetic acid (EDTA) cannot remove it. *P. woesei* α-amylase was shown to be inhibited by high Ca^{2+} concentrations [29]. Ca^{2+} can bind some catalytic residues [42], and therefore can inhibit α-amylase activity. This observation could explain the inhibition of the *P. woesei* enzyme at high Ca^{2+} concentrations.

Chung et al. have shown that *T. profundus* α-amylase remained fully active after dialysis against an EDTA-containing buffer [37]. The same was observed with the *Pyrococcus* sp. KOD1 and *P. furiosus* extracellular α-amylases [19,40]. However, inhibition of *P. furiosus* extracellular α-amylase by EDTA and ethyleneglycotetraacetic acid (EGTA) has been observed by Brown et al. [39]. These apparently conflicting results are probably due to the fact that these two teams used different enzymatic preparations. The fact that EDTA does not inhibit some of these enzymes suggest that Ca^{2+} and divalent metallic cations are not involved in stabilizing these enzymes. Nevertheless, the fact that *P. woesei* α-amylase is inhibited by EDTA [29] shows that this conclusion is not true for all archaeal α-amylases.

The effects of other metals on thermophilic archaeal α-amylase activities are metal-specific and depend on the enzyme source (Table 3). Three effects have been observed: 1) an inhibition; 2) an activation of the enzymatic activity; or 3) no significant effect.

It has been shown that *P. furiosus* extracellular α-amylase was not activated by any of the metals tested (Ca^{2+}, Mg^{2+}, and Mo^{2+}) [40]. On the other hand, the same enzyme was partially inhibited by Co^{2+}, Ni^{2+}, and Fe^{2+} (2 mM), and almost completely inhibited by Cr^{3+}, Zn^{2+}, and Cu^{2+} (2 mM) [40]. As far as *P. furiosus* intracellular α-amylase is concerned, inhibition was observed with Co^{2+}, Cr^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, and Zn^{2+} [30].

P. woesei α-amylase was activated by 1 mM Ca^{2+}, 1 mM Co^{2+}, and 3 mM Mo^{2+}, poorly inhibited by 2 mM Mg^{2+} and 3 mM Ni^{2+}, and almost completely inactivated by 1 mM Cr^{2+}, 1 mM Cu^{2+}, and 2 mM Zn^{2+} [29].

Pyrococcus sp. KOD1 α-amylase is completely inhibited by 1 mM Cu^{2+} or Zn^{2+} [19]. Approximately 50% of the activity is lost in the presence of Co^{2+} and Mn^{2+}. Other metals had no effect on this enzyme activity. Hg^{2+} completely inhibited *T. profundus* α-amylase activity, whereas Ba^{2+}, Cu^{2+}, Cd^{2+}, and Zn^{2+} (1 mM) reduced its activity by half [37]. Ca^{2+}, Cs^{2+}, and Mg^{2+} moderately activated this enzyme, whereas Co^{2+}, Fe^{2+}, and Sr^{2+} had no effect.

T. hydrothermalis α-amylase was activated by Ca^{2+}

Table 4
Mainly produced maltodextrins by the action of thermophilic archaeal α-amylases on starch

Enzymes	Maltodextrins	References
Extracellular α-amylases		
Pyrococcus furiosus	Maltoheptaose, Maltohexaose, Maltotetraose	18
Pyrococcus woesei	Maltopentaose, Maltose	29
Pyrococcus sp. KOD1	Maltotriose, Maltose	19
Thermococcus hydrothermalis	Maltotetraose, Maltotriose	21
Thermococcus profundus	Maltotriose, Maltose	37
Intracellular α-amylase		
Pyrococcus furiosus	Maltohexaose, Maltopentaose, Maltotetraose	30

[21]. Cu^{2+} had no effect on this enzyme activity, even at concentrations up to 5 mM. *T. hydrothermalis* α-amylase was slightly inhibited by Ni^{2+} (17% inhibition by 5 mM Ni^{2+}) and more significantly by Mg^{2+} (45% inhibition by 5 mM Mg^{2+}) and Mn^{2+} (98% inhibition by 1 mM Mn^{2+}). However, Mn^{2+} concentrations above 1 mM were not as inhibitory (only 73% inhibition by 5 mM Mn^{2+}). A similar observation was made for the *P. woesei* α-amylase with magnesium (partial inactivation by 2 mM Mg^{2+} and activation by 3 mM Mg^{2+}) [29]. No explanation of these phenomena was given by these authors. What often occurs is that the enzymes have multiple metal-binding sites with varying affinity for different metals. A metal present at high concentrations might compete with another metal present at lower concentration and replace it in a metal-binding site, even if its affinity for the binding site is lower leading to a modification of the enzymatic activity.

According to Irving–Williams order [43], from Ba^{2+} to Zn^{2+}, the ionic radius decreases and the ionization potential increases. This correlates with the fact that almost all of these enzymes are inhibited by Zn^{2+} and Cu^{2+} (Table 3), which are at the opposite end of the Irving–Williams order with respect to Ca^{2+}.

4.3. Starch-degradation products obtained with thermophilic archaeal α-amylases

Products obtained from starch hydrolysis by thermophilic archaeal α-amylases differ from enzyme to enzyme and depend on the hydrolysis conditions (substrate concentration, incubation time, enzyme, etc.). For short incubation times, the main products are always dextrins. This is due to the endo-acting nature of these α-amylases which are classified as liquefying enzymes [17,21]. If reaction times are increased, the initially formed dextrins are hydrolyzed to maltodextrins. The main maltodextrins produced depend on the enzyme source (Table 4).

5. Archaeal α-glucosidases

5.1. Generalities

α-Glucosidases (α-D-glucoside glucanohydrolase; EC 3.2.1.20) hydrolyze the terminal nonreducing α-1,4 linkage in a variety of substrates, including disaccharides, oligosaccharides, and other aryl-and alkyl-α-glucopyranosides, producing α-D-glucose. They are involved in the last step of starch degradation. They are typically inactive on high-molecular-weight substrates such as starch or pullulan. Only a few archaeal α-glucosidases have been described: from *P. furiosus*, *P. woesei*, *Sulfolobus shibatae*, *S. solfataricus*, *T. hydrothermalis*, and *Thermococcus zilligii* [39,44–49]. Only the *S. solfataricus* maltase and the *T. hydrothermalis* α-glucosidase encoding genes have been cloned [50,51]. Nevertheless, the cloned α-glucosidase gene from *T. hydrothermalis* [51] encodes an enzyme that differs from that already purified from this Archaea by Legin et al. [49].

5.2. Biochemical properties

Molecular weights of thermophilic archaeal α-glucosidases greatly differ from each other: 60 kDa for the enzyme of *T. zilligii*, 80 kDa for that of *S. solfataricus* and 125 kDa for the *P. furiosus* α-glucosidase [44,47,48]. According to the method used, *T. hydrothermalis* α-glucosidase shows a molecular mass of 57 kDa (gel filtration) or 116 kDa (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) [48]. Nevertheless, the fact that this enzyme is not denatured in the presence of sodium dodecyl sulfate, but activated instead [49], indicates that the results obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis must be analyzed with caution. According Di Lernia et al. [46], the *S. shibatae* α-glucosidase (MW 313 kDa) seems to be a tetramer composed of subunits similar or identical to the other *S. solfataricus* α-glucosidase. With optimal temperatures for activity above 100°C, archaeal α-glucosidases are highly thermophilic, and their optimal pH for activity is rather acidic (Table 5). All of them are intracellular enzymes, with the exception of *T. zilligii* α-glucosidase, which has been described as extracellular [48]. This enzyme appears different from the other Thermococcale α-glucosidases: it is the only extracellular enzyme, and, in contrast to the other Thermococcale α-glucosidases, it has an alkaline optimal pH for activity.

α-Glucosidases can be classified according their substrate preference: Type I have a preference for *p*-nitrophenyl-D-glucopyranoside and Type II have a preference for maltooligosaccharides. Thermococcale α-glucosidases are Type I α-glucosidases, whereas the crenarchaeotal *S. solfataricus* enzyme is a Type II α-glucosidase (maltase) [47].

Few studies were made on the impact of different agents and ions on archaeal α-glucosidase activities. Piller et al. have shown that the *T. zilligii* enzyme is inhibited by Mg^{2+}, Ca^{2+}, Zn^{2+} and more significantly by Co^{2+} and Ni^{2+} [48].

Table 5
Some biochemical characteristics of thermophilic archaeal α-glucosidases

Archaebacteria	pHmin[a]	pHopt	pHmax[a]	Tmin[a]	Tmax[a]	Topt[a]	Molecular weight (kDa)[a]	α-glucosidase type[a]	Localization[b]	References
P. furiosus	nd	5.0–6.0	nd	60	105–115	nd	125	I	i	44
P. woesei	nd	5.0–5.5	nd	nd	105	nd	nd	nd	i	45
S. shibatae	3.5	5.5	9.5	40	85	nd	313	nd	i	46
S. solfataricus	3.0	4.5	8.0	25	105	>120	80	II	i	47
T. hydrothermalis	4.5	5.5	9.0	40	110	120	57	I	i	49
T. zilligii	3.5	7.0–9.0	nd	nd	nd	nd	60	I	e	48

[a] nd, not determined by the authors.
[b] i, intra-cellular enzyme; e, extra-cellular enzyme.

Mercury ions completely inhibit this enzyme. On the other hand, EDTA-treatments increase this enzyme activity, presumably by removing trace heavy metals. *T. hydrothermalis* α-glucosidase is inhibited by EDTA and ethyleneglycotetraacetic acid, as well as by the nonionic detergent triton X-100 [49]. Ca^{2+}, Mg^{2+}, and Mn^{2+} have no effect, whereas Cu^{2+} and Fe^{2+} are inhibitory. As for the *T. zilligii* α-glucosidase, Hg^{2+} completely inactivates the *T. hydrothermalis* α-glucosidase.

6. Archaeal pullulanases

6.1. Generalities

Pullulanases (pullulan α-glucano-hydrolase; EC 3.2.1.41) cleave the α-1,6 glucosidic bonds in pullulan, producing maltotriose. Depending on their inability or ability to degrade α-1,4 glucosidic linkages in other polysaccharides, they are classified in two categories: Type I and Type II, respectively. Type II pullulanases are also called amylopullulanases. Because pullulanases are able to hydrolyze the α-glucosidase-resistant α-1,6 linkages found in dextrins, they are used in combination with α-glucosidases in starch saccharification (Fig. 1), improving the saccharification rate and yield. The first thermophilic archaeal pullulanase described was the *P. furiosus* enzyme [39]. Similar enzymes have since been described from *Desulfurococcus mucosus*, *P. woesei*, *T. aggregans*, *T. celer*, *T. guaymagensis*, *T. hydrothermalis*, *Thermococcus litoralis*, strain ES4 [38,52–56], and from numerous other Thermococcale strains [14,57]. The genes encoding the *P. woesei*, *P. furiosus*, and *T. hydrothermalis* enzymes have been cloned [55, 58,59]. All these enzymes are Type II pullulanases. No Type I pullulanase has been characterized from thermophilic Archaea. These enzymes seem to be very rare among thermophiles in general. [60]

6.2. Biochemical properties

It has been shown that some archaeal pullulanases are glycoproteins [52,53] with molecular weights above 100 kDa. However, *P. woesei* pullulanase (90 kDa) seems to switch to an extreme compact and rigid form (45 kDa) at temperatures below 80°C [55]. Schuliger et al. have shown that strain ES4's pullulanase exhibits two bands of 110 kDa and 140 kDa after sodium dodecyl sulfate-polyacrylamide gel electrophoresis [53]. This could be due to the presence of two enzymes with similar enzymatic activity or to the presence of two versions of the same enzyme modified by glycosylation or truncated. With the exception of the two enzymatic forms found in strain ES4 that are optimally active at 110°C and 125°C [53], archaeal pullulanases are typically optimally active between 80°C and 100°C and between pH 5.5 and 6.5 (Table 6).

Few studies were made on the effect of divalent ions on the activity of thermophilic archaeal pullulanases. However, it has been shown that Ca^{2+} not only has a positive effect on the activity of these enzymes and that it can also increase their thermostability and/or their optimal and maximal temperatures of activity [52,55,56,58]. For example, *P. furiosus* pullulanase has its optimal and maximal temperatures of activity increased respectively from 98°C to 130°C and from 120°C to 140°C in the presence of 5 mM Ca^{2+} [52]. These authors also showed that Sr^{2+} and Mg^{2+} were, respectively, able to activate and reduce the thermoactivity of *P. furiosus* and *T. litoralis* pullulanases. Other studies showed that the *P. furiosus* enzyme was activated by Ca^{2+} and Sr^{2+}, whereas Cu^{2+}, Ni^{2+}, and Zn^{2+} were strong inhibitors [58]. *T. hydrothermalis* pullulanase activity is not affected by Mg^{2+}, but it is slightly increased by Mn^{2+} [56]. However, Ni^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} are strong inhibitors. On the other hand, Rüdiger et al. have shown that Mg^{2+}, Mn^{2+}, and Co^{2+} do not affect *P. woesei* pullulanase activity but also that this enzyme is completely inhibited by Ni^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Cr^{2+} [55]. *T. hydrothermalis* pullulanase is inhibited by EDTA [56]. Brown and Kelly did not observe such an inhibition for the *P. furiosus* enzyme [52], in spite of having previously shown that it was not only inhibited by EDTA but also by ethyleneglycotetraacetic acid [39]. This discrepancy could be due to the fact that these authors have used preparations with different levels of purity: crude extract [39] and purified enzyme [52]. On the other hand, Dong et al. [58] have

Table 6
Some biochemical characteristics of thermophilic archaeal pullulanases

Archaebacteria	pHmin[a]	pHopt	pHmax	Tmin[a]	Topt[b]	Tmax[b]	Molecular weight (kDa)[a]	Pullulanase type[a]	References
D. mucosus	nd	5.0	8.0	nd	100	115	nd	nd	38
P. furiosus	4.3	5.5	7.8	60	98 (130)	130 (140)	110	II	52
P. woesei	5.0	5.5–6.0	9.5	50	100	120	90	II	55
T. aggregans	nd	6.5	9.0	60	100	115	nd	nd	38
T. celer	nd	6.0	9.0	60	90	120	nd	nd	38, 54
T. guaymagensis	nd	5.5	8.5	60	100	120	nd	nd	38
T. hydrothermalis	3.5	5.5	9.0	40	95 (110)	110 (120)	110–128	II	56
T. litoralis	4.3	5.5	7.8	60	98 (120)	120 (130)	119–125	II	52
strain ES4	4.0	5.5–6.5	7.5	80	110–125 (120)	130 (145)	110–140	II	53

[a] nd, not determined by the authors;
[b] Values in brackets indicate the Topt and the Tmax measured in presence of calcium.

shown, for the recombinant form of the *P. furiosus* pullulanase produced by *E. coli*, that after extensive EDTA-treatment this enzyme had three times less activity than in the presence of Ca^{2+}. Moreover, no information is available on the three-dimensional structure of the *P. furiosus* pullulanase and this enzyme displays a lack of similarity with other amylolytic enzymes with a known structure [58]. This does not allow us to draw any conclusion about mechanism by which Ca^{2+} stabilizes and activates this enzyme.

7. Archaeal α-amylase structure and phylogenetic relationships with the other α-amylases

Alignments of the thermophilic archaeal α-amylase protein sequences have shown that Thermococcale enzymes, with the exception of the *P. furiosus* intracellular α-amylase, are highly similar (>75% similarity) (Fig. 3) [21]. Only the *T. profundus* sequence possesses a lesser degree of homology with the others. That is due to the fact that this later is shorter than the other Thermococcale secreted α-amylases (Fig. 3): only 401 amino acids compared with other Thermococcale enzymes contain 457 to 469 residues [17–23]. One hypothesis concerning the *T. profundus* α-amylase length is that the authors made a sequencing mistake that changed the reading frame and the size of the protein. No one has been able to produce α-amylase mutants with C-terminal deletions that would remain active. The C-terminal domain seems to be essential for α-amylase activity.

We recently compared the *T. hydrothermalis* α-amylase sequence with those of 21 other α-amylases from the three phylogenetic kingdoms (Eucarya, Eubacteria, and Archaea) which were representative of more than 100 known α-amylase sequences [61]. This study allowed us to show that this enzyme, as well as the other Thermococcale secreted α-amylases, contain most of the β-strands and the pentapeptide stretch located near the C-terminus of loop $\beta3 \rightarrow \alpha3$ that are present in family 13 glycoside-hydrolases [62]. These results indicate that Thermococcale secreted α-amylases belong to glycoside-hydrolases family 13 [21]. On an other hand, *P. furiosus* intracellular α-amylase does not contain these conserved regions, and it belongs to glycoside-hydrolases family 57 instead. Moreover, we recently showed that the α-amylase from the methanogenic Archaeon *Methanococcus jannaschii* contains features of both α-amylase families, indicating that these two glycoside-hydrolase families are either the products of a very distant common ancestor or that one evolved from the other [63].

We have also shown that, in the conserved regions, Thermococcale α-amylases show some characteristic sequence features [21,61]. In strand β4 in particular, a tryptophan is present in all the archaeal enzymes, and a histidine, supposed to be involved in substrate binding [64], is replaced by a glycine. Lee et al. suggested that the tryptophan found in strand β4 could be involved in catalysis in replacement of the missing histidine [20]. From the sequence alignment of the α-amylases of the three phylogenetic domains, an unrooted phylogenetic tree has been drawn (Fig. 4) [21,61]. This tree clearly demonstrates the evolutionary relatedness of archaeal and plant α-amylases. Similar results have been obtained by Matsui et al. (unpublished data) for β-glucosidases (enzymes belonging to glycoside-hydrolases family 1 and adopting an $(\alpha/\beta)_8$-barrel structure). Interestingly, *S. solfataricus* α-glucosidase (in glycoside-hydrolases family 31) has also been clustered together with its eukaryotic (i.e. plant, fungal, and mammalian) counterparts [50]. Our α-amylase tree also shows that archaeal α-amylases are closer to the liquefying α-amylases, represented by the *B. licheniformis* enzyme, than to the saccharifying ones, represented by the *B. subtilis* and *L. amylovorus* enzymes. This result agrees with the nature of the products of starch hydrolysis by the archaeal enzymes, which showed that archaeal α-amylases were liquefying enzymes.

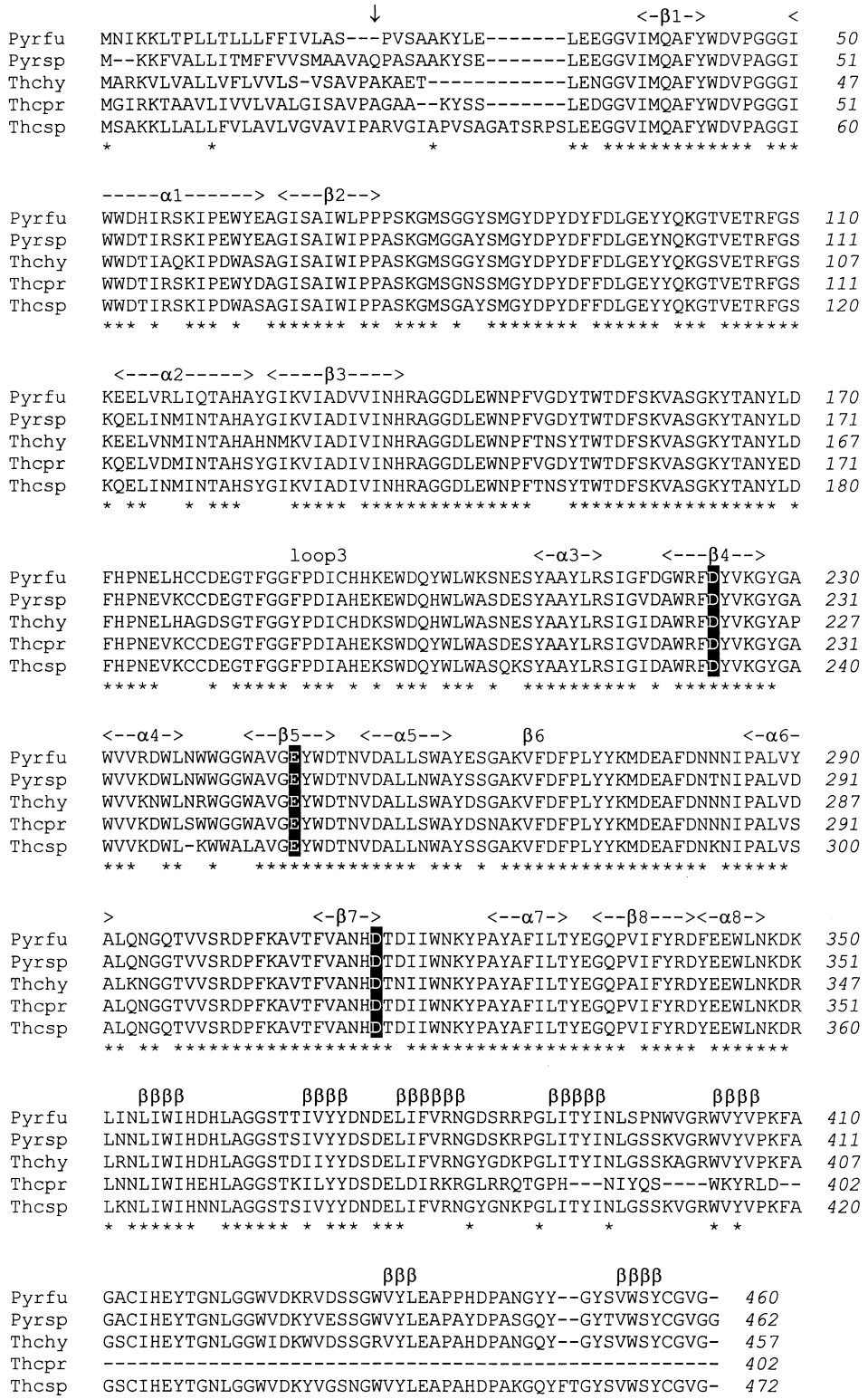

Fig. 3. Amino acid sequence alignment of archaeal α-amylases. Enzyme sources are abbreviated as: Pyrfu, *Pyrococcus furiosus* strain DSM 3638 [16–18]; Pyrsp, *Pyrococcus* species strain KOD1 [19]; Thchy, *Thermococcus hydrothermalis* strain AL662 [21,22]; Thcpr, *Thermococcus profundus* strain JCM9378 [20]; and Thcsp, *Thermococcus* species strain Rt3 [23]. Sequences are numbered from the N-terminus including the signal peptides. The start of the mature enzymes is indicated by a vertical arrow. Identical residues and gaps are represented by asterisks and dashes, respectively. Secondary structure elements predicted for the $(\alpha/\beta)_8$-barrel and the C-terminal β-sheet domain by the GOR method [66] are shown above the alignment. The short conserved sequence located near domain B's C-terminus is indicated as "loop3" above the alignment. The three proposed catalytic residues (Asp, Glu, and Asp in strands β4, β5, and β7, respectively) are highlighted by black rectangles.

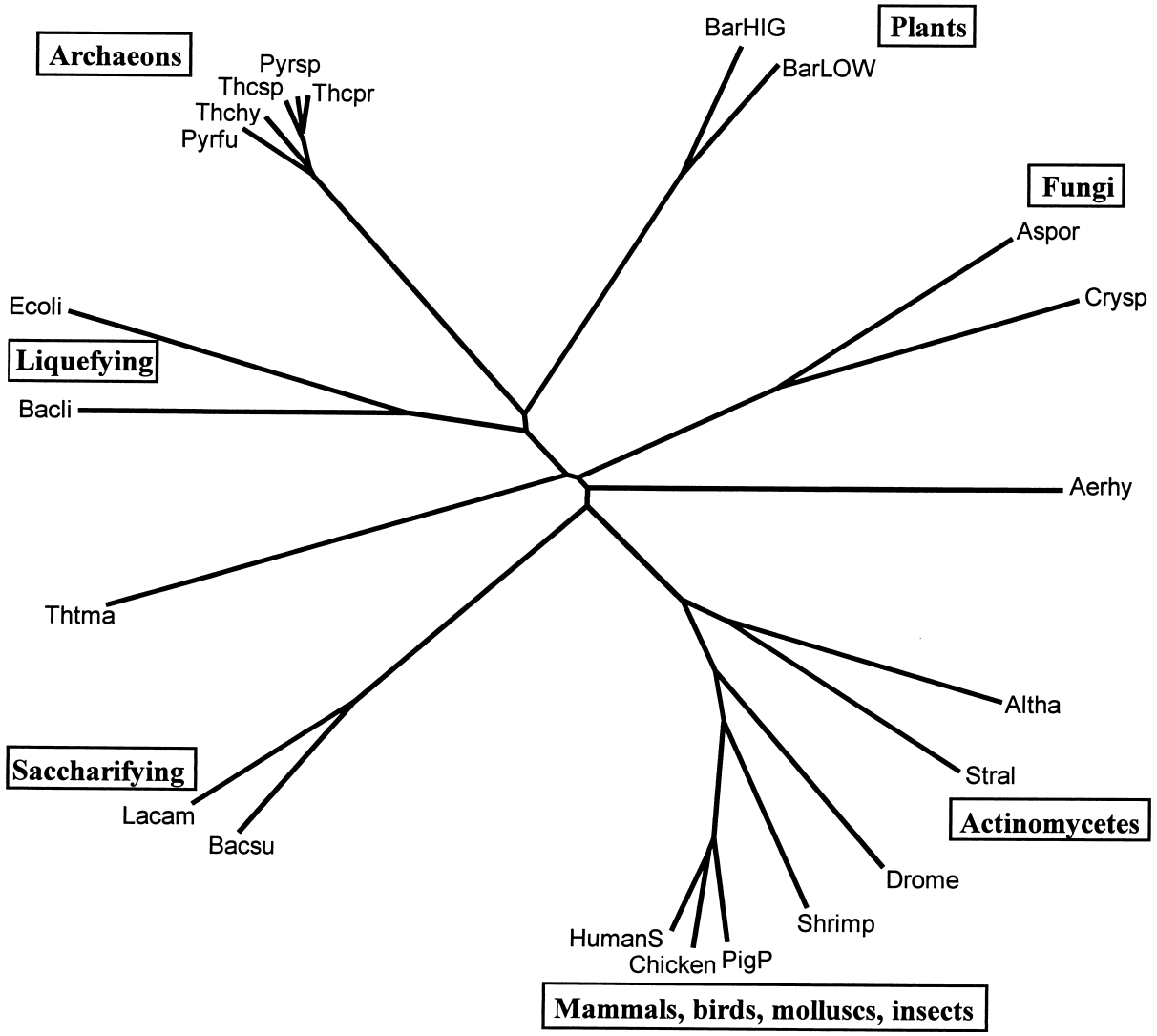

Fig. 4. α-Amylase evolutionary tree [21]. Only representative from eubacterial, eukaryal and archaeal enzymes are included. This tree is based on a sequence alignment starting at strand β2 and ending at strand β8 of the $(\alpha/\beta)_8$-barrel and including the entire B domain (i.e. loop β3→α3). The branch lengths are proportional to the divergence of the sequences of the individual α-amylases. The sum of the lengths of the branches linking any two α-amylases is a measure of the evolutionary distance between them. The α-amylase sources are abbreviated as follows: Aerhy, *Aeromonas hydrophila*; Altha, *Alreromonas haloplanctis*; Bacli, *Bacillus licheniformis*; Bacsu, *Bacillus subtilis*; Ecoli, *Escherichia coli*; Lacam, *Lactobacillus amylovorus*; Stral, *Streptomyces albidoflavus*; Thtma, *Thermotoga maritima*; Pyrfu, *Pyrococcus furiosus*; Pyrsp, *Pyrococcus* sp. Rt-3; Thchy, *Thermococcus hydrothermalis*; Thcpr, *Thermococcus profundus*; Aspor, *Aspergillus oryzae*; Crysp, *Cryptococcus* sp.; BarHIG, Barley (high pI isozyme); BarLOW, Barley (low pI isozyme); Drome, *Drosophila melanogaster*; Chicke, Chicken; HumanS, Human (saliva); PigP, Pig (pancreas); Shrimp, Shrimp.

8. Conclusion

Thermophilic Archaea express various enzymes, including amylolytic enzymes (i.e. α-amylases, α-glucosidases, and pullulanases) that are naturally thermophilic. Their biochemical properties allow these enzymes to work together in similar conditions of pH and temperature. These enzymes are interesting candidate catalysts for testing a one-step process for the industrial bioconversion of starch into glucose. A one-step process that used these efficient and thermoactive enzymes would decrease glucose production costs. Thermophilic archaeal amylolytic enzymes have not been introduced into current industrial processes. Archaeal α-amylases, in particular, have to compete with the *B. licheniformis* and *B. stearothermophilus* enzymes that already have excellent thermophilic properties. Nevertheless, it does not let us think about the possible use of thermophilic archaeal amylolytic enzymes in new biotechnological applications.

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