Cah. Biol. Mar. (2002) 43 : 409-412

# Biosynthesis of a reserve endopolysaccharide in the hyperthermophilic archaeon *Thermococcus hydrothermalis*

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## Introduction

The biosynthesis of reserve endopolysaccharides such as starch and glycogen is well spread amongst living organisms (François et al., 2001; Preiss, 1988, 1984). Starch is produced by the chloroplast of photosynthetic organisms, whereas glycogen is synthesized by most of Eubacteria and non-photosynthetic Eukarya. Both are polymers of glucose, with the same average structure, α-1,4 linked, with punctual interchains ramifications in α-1,6 positions. Starch is constituted of two distinct families of macromolecules: amylopectin and amylose, organized in a very highly structured complex within the granule. Amylopectin, the major fraction, is highly ramified (5-6%) and possesses fairly long α-1,4 glucanic branching chains containing up to 20 glucose residues, depending on the organism. Amylose, the minor fraction, is constituted of very long α-1,4 glucanic chains with occasional branching points (less than 1%) (Myers et al., 2001). On the contrary, glycogen is not very structured (Sandhyarani et al.,1992), more ramified than amylopectin (between 8 to 10% of α-1,6 linkages), and possesses very short α-1,4 glucanic chains, which does not exceed a Degree of Polymerisation of 7 glucose residues (DP=7). Though starch and glycogen are very different, their biosynthetic pathways are similar. They begin with the transformation of a glucose residue into glucose-1-phosphate, which is transformed into glucose-6-phosphate and then into Adenosine-5'-DiPhosphoGlucose (ADPG), for Eubacteria and photosynthetic Eukarya, and Uridine-5'-DiPhosphoGlucose (UDPG), for non-photosynthetic ones. These molecules serve as substrates for starch or glycogen synthases (or ADP-glucose, or UDP-glucose): α-1,4-D-glucan-α-4-glucosyltransferase EC 2.4.1.21). These enzymes transfer the glucose residue from the glucosylnucleotide to the non-reducing end of an α-1,4 glucan. This elongation step is followed by a ramification step: the branching enzymes (α-1,4-D-glucan: α-1,4-D-glucan-6-glucosyltranferase EC 2.4.1.18) cleave the α-1,4 glucans and transfer them into an α-1,6 position. Depending on the living domain, elongation and ramification activities will implicate one or many isoforms (Farkas et al., 1991; François et al., 2001; Laarson et al., 1996 ; Mirta, 1998). These two last steps are absolutely characteristic of the endopolymer biosynthesis. Previous work has reported the existence of this metabolic pathway in the Archaea domain, which looked very similar to the one described for the other Prokarya. The elongation activity has been partially purified in the Crenarcheota *Sulfolobus acidocaldarius* Brock et al., 1972 (König et al., 1982) and in the Euryarcheota *Thermococcus hydrothermalis* Godfroy et al., 1997 (Gruyer et al., 2002). The first archeal branching activity was also isolated in the latter strain. However, the polymers accumulated by these micro organisms are not similar: Glycogen was purified from *Sulfolobus acidocaldarius* (König et al., 1982), whereas *Thermococcus hydrothermalis* produced, at the end of the exponential phase, an endopolysaccharide built with longer α-1,4 glucanic chains that was called "amylopectin-like" (Gruyer et al., 2002). However, further investigation performed on the same reserve endopolysacharide produced by *Thermococcus hydrothermalis* during the log phase led to a glycogen-like structure, suggesting an important structural reorganization of the reserve endopolysaccharide at the end of the log phase. In this paper we wish to present the purification and characterization of this glycogen-like structure produced by *Thermococcus hydrothermalis* during the exponential growth phase as well as some characteristics of elongation

and branching activities. These results indicate a relationship between *Sulfolobus* and *Thermococcus* species.

## Material and methods

*Bacterial strain and culture conditions*
*Thermococcus hydrothermalis* AL662$^T$ (CNCMI-1319) (Godfroy et al., 1997) was cultivated anaerobically as previously described (Legin et al., 1998) at 85 °C in a fermentor using BS medium (pH 6.0) with 0.4% maltose instead of 0.4% starch.

*Extraction of the intracellular content*
The cells were harvested by centrifugation and resuspended in 200 mM phosphate buffer (pH 6.0), containing 0.01% $NaN_3$. The concentrated suspension was subjected to a French Press (52.2 MPa) and centrifuged for 3 h at 10000 *g*, at 4 °C. The crude extract was stored at 4 °C for further enzymatic experiments and at -20 °C for endopolysaccharides analysis.

*Analysis of the endopolysaccharide accumulated by* Thermococcus hydrothermalis
The amount of polysaccharide was determined by the enzymatic assay kit for starch determination (Diffchamb). The endopolymer was purified as previously described by Gruyer et al. (2002) by successive proteolysis, ultrafiltrations and precipitations methods.
NMR spectra. The purified fraction was freeze-dried and exchanged twice with 500 µl of Sigma ultra-pure deuterium oxide (99.96%). The freeze-dried sample was dissolved into 99.9% deuterated DMSO (Sigma)/deuterium oxide (5:1) and incubated for 1 h at 80 °C before analysis. Proton NMR spectroscopy measurements were recorded at 80 °C on a 500 MHz DRX500 Bruker spectrometer in 5 mm tubes (polymer concentration: 2 mg ml$^{-1}$). Proton spectra were obtained with 1072 scans per experiment; relaxation delay of 2 sec between scans. Deuterated DMSO was used as an internal standard reference at 2.65 ppm.

*Enzymatic preparations and assays*
Elongation activity was partially purified after two anions exchange chromatography steps (DEAE Sephadex – Amersham Pharamacia Inc.) (Gruyer et al., 2002) and assayed as follows by using radiolabeled ADPG and UDPG and commercial glycogen (from Rabbit liver – Sigma) as substrates (Mouille et al., 1996): Sample (20 µl of semi-pure fraction) was assayed at 85 °C for 25 min by using 80 µl citrate-phosphate buffer (0.1 M, pH 5.5) containing 2.66 µM ADPG [$^{14}$C] (25 µCi ml$^{-1}$; 200 mCi mmol$^{-1}$) or 9.2 µM UDPG [$^3$H] (1 mCi ml$^{-1}$; 100 Ci mmol$^{-1}$) as already described (Fontaine et al., 1993). The pH for the optimal activity was measured at 85 °C between pH 4.0 and pH 7.5 using 0.1 M citrate-phosphate and TRIS-HCl buffers. The optimal temperature was determined between 25 °C and 110 °C with 0.1 M citrate-phosphate buffer pH 5.5.

Branching activity was detected at 40 °C by "phosphorylase stimulation" zymograms and isolated after protein electroelution as previously described (Fontaine et al., 1993).

Branching activity was assayed following the branching linkages assays (Takada et al., 1994): amylose 0,555% was branched by incubation at 80 °C with the electro-eluted samples, containing the previously detected branching activity. The branching linkages assay consists in the measurement of the reducing sugars liberated after enzymatic disbranching (using 4 units of commercial isoamylase - Hayashibara Inc.) of newly formed branches.

## Results

*Biosynthesis activities of reserve endopolysaccharide*
Elongation activity from *T. hydrothermalis* was characterized after the elimination of all the endopolysaccharide degradation activities. The molecular mass of the enzyme was calculated to be about 42 kDa by SDS-PAGE and 85 kDa ± 5 kDa by gel filtration. This data suggests that the enzyme could be constituted of two subunits. The partially purified enzyme was optimally active at pH 5,5 and at a temperature of about 85 °C (Fig. 1). The glycosyltransferase was thermostable for at least 2 hours at 80 °C. This enzyme was also able to use ADPG and UDPG as substrate with the same affinity but with a 10-fold better catalytic rate for ADPG (Gruyer et al., 2002).

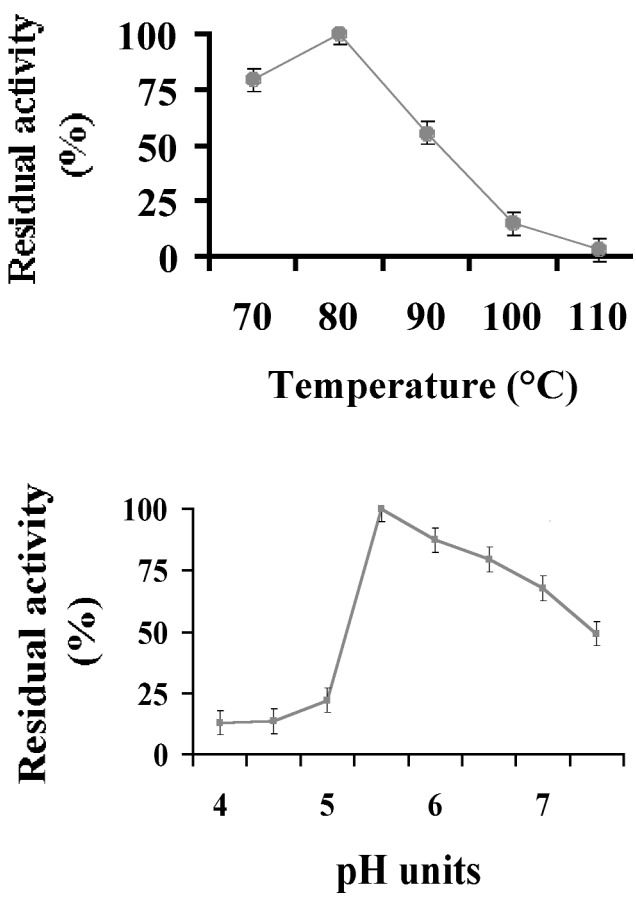


**Figure 1.** Elongation activity as a function of **A.** temperature and **B.** pH.

The branching activity was detected by zymograms and a 65 kDa protein (SDS-PAGE) was isolated. The branching enzyme was particularly difficult to study because of its instability: An substantial loss of activity, together with a decrease of electrophoretic mobility, as detected on SDS-PAGE (Molecular Mass estimated to be about 40 kDa), was observed after only few days at 4 °C with semi purified samples and after 4 weeks with crude intracellular extract (data not shown). This instability may be due to the loss of stabilizing molecules. We have shown that even at 4 °C, intracellular crude extracts still had enough residual enzymatic activity to allow a total degradation of the glucose endopolymer: The amount of endopolysaccharide could reach a maximum of 1.1 mg $ml^{-1}$, and it completely disappeared after 1 month at 4 °C. For this reason, it was fundamental to store the intracellular contents at -20 °C in order to inactivate enzymatic activities.

The branching activity was optimal between 70 °C and 80 °C (Fig. 2) and in a broad range of pH (4<pH<7).

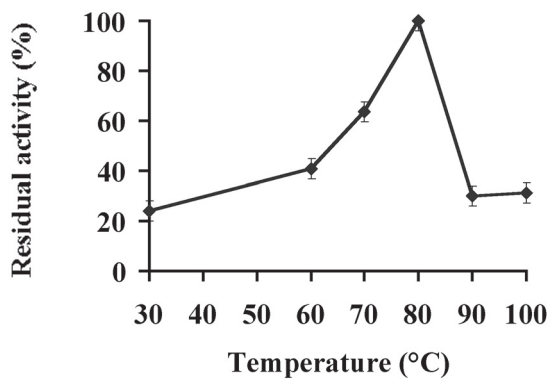


**Figure 2.** Branching activity as a function of temperature: Semi-purified enzyme (100 µl) was incubated with 0.555% amylose (900 µl) at 80 °C for 30 minutes. The branching linkages assays were performed as described by Takata et al., 1994.

*The reserve endopolysaccharide accumulation in* T. hydrothermalis

As for all other Archeal microorganisms, *Thermococcus hydrothermalis* only accumulates a reserve endo-polysaccharide during the exponential growth phase. This polysaccharide is then quickly degraded during the stationary phase (Preiss, 1984). Various analyses were performed with a 4-hour culture, when the amount of polymer in the cell was at its maximum. The endopolysaccharide presented an average chain length of 14 with a branching rate of 7,5% and was then called "amylopectin-like" (Gruyer et al., 2002).

A study of the polymer was also performed on a 3-hour culture in order to determine the polysaccharide structure during its biosynthesis. Unlike the 4-hour culture polymer, this polymer was not retained by a 100 kDa ultra filtration, indicating a smaller molecular mass. The proton NMR spectra of the polymer purified at 75% of the exponential

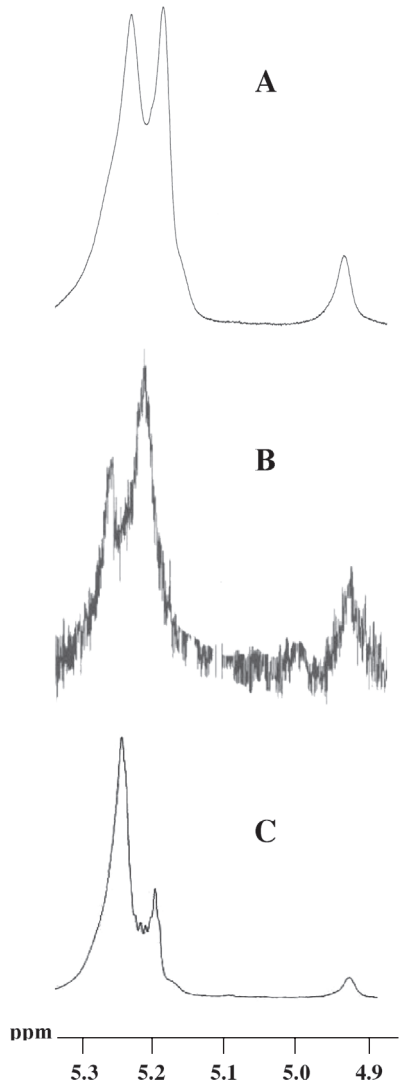


**Figure 3.** [$^1$H] NMR analysis of different endopolysaccharide. **A.** Oyster Glycogen; **B.** the endopolysaccharide purified from *T. hydrothermalis* after 3 hours of cultures; **C.** *Waxy* cultivar Amylopectin.

growth indicated the prevalence of short α-1,4 glucanic chains as in glycogen. As shown in Fig 3 B, the importance of the 5.2 ppm peak as compared to the 5.3 ppm peak allows short chain structures (e.g. glycogen A) to be distinguished from long chain ones (e.g. amylopectin C) (Colleoni et al., 1999 ; Mouille et al., 1996). The branching rate was estimated by integration of the 4.95 ppm peak (Fig 3 B) to be about 8 ± 0.5 %.

## Discussion

Biosynthesis of reserve polysaccharide is widely spread in living organisms and the various steps involved in theses biosyntheses are very similar. Eubacteria only possess one ADP glucose-using elongation enzyme and only one

branching enzyme. Similarly *Thermococcus hydrothermalis* only has one ADP glucose-using elongation enzyme. The branching activity, though very difficult to identify, may involve only one enzyme. Therefore our studies on *Thermococcus hydrothermalis* suggest homogeneity and a similarity in the biosynthetic pathway of the reserve endopolysaccharides within the prokaryotes.

However, the polysaccharide accumulated by *Thermococcus hydrothermalis* appears to be very peculiar: Until now, the only described archaeal reserve endopolysaccharide was glycogen (König et al., 1982). However, these studies did not specify at what stage of the microbial growth the polymer was purified. Our studies have shown that *T. hydrothermalis* produced an endopolymer whose structure appeared to be changing with the physiological cellular states. During the accumulation phase (3 hour of culture), the polysaccharide was similar to glycogen, but the ultimate structure, obtained from the end of this phase (4 hour of culture) was "amylopectin-like". This last very ephemeral structure is quickly degraded after the beginning of the stationary phase.

It would be interesting to understand the reasons for this structural modification in the reserve endopolysaccharide at the end of the exponential growth phase. Further investigations are being performed in order to answer this question, but these data suggest that other enzymatic activities than elongation and branching could be involved in reserve endopolysaccharide biosynthesis during this transition at the end of the log phase.

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