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Expression and characterisation of the catalytic domain of an archaeal family 57 pullulanase type II

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The catalytic domain (ApuΔ2) of the thermostable family 57 pullulanase type II from the archaeabacterium *Thermococcus hydrothermalis* has been cloned. The activity of ApuΔ2 in the presence of various reagents has been characterised and the effects of pH and temperature have been determined. Our results show that ApuΔ2 possesses an extreme thermostability, identical to that of the wild type parent enzyme, which may be mainly due to electrostatic interactions at the protein level. Furthermore, data indicate that this thermostability is increased in the presence of calcium and in the presence of substrates, and particularly non-reducing malto-oligosaccharides. Finally, although N-bromosuccinimide treatment leads to the indiscriminate inactivation of both activities, pH dependence and acarbose inhibition data have revealed differences in the behaviour of ApuΔ2 towards α-1,6 and α-1,4 glucosidic linkages. Therefore, overall these results suggest that ApuΔ2 may possess two active sites. Using a combination of spectroscopic methods and secondary structure predictions, the ApuΔ2 secondary structure has been examined. Good agreement between the experimentally-adjusted predictions has allowed the generation of a high quality consensus prediction which indicates that ApuΔ2 may be composed of several distinct structural elements. Notably, this analysis has allowed the identification of a major region (amino acids 127 to 578) which exhibits an alternating pattern of β-strands and α-helices, which is highly reminiscent of the $(\alpha\beta)_8$ fold which is commonly associated with family 13 amylolytic enzymes.

Key words: pullulanase, F-57, glycosyl hydrolase, thermostable, *Thermococcales*, acarbose.

Abbreviations: F-13, F-15 and F-57, glycosyl hydrolase family 13, 15 and 57, respectively; Th-Apu, pullulanase type II from archaebacterium *Thermococcus hydrothermalis* AL662; ApuΔ2, catalytic domain of Th-Apu.

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Introduction

Since the 1960's the starch industry has employed an exclusively enzymatic technology for the commercial scale production of glucose syrup. However, because the current process conditions for starch liquefaction and saccharification were originally elaborated to suit the available enzymes, these processes are far from perfect. Therefore, for some years now, researchers have sought to improve the liquefaction and saccharification steps either by adapting the existing enzymes by genetic engineering (Ford, 1999; Shaw et al., 1999) or by replacing them with new, more suitable ones. So far, the first option appears to be the one preferred by the starch industry, possibly because it is more expedient, while the search for new enzymes has been mainly performed by independent research groups.

One of the major objectives for workers searching for new amylolytic enzymes is the identification of highly thermostable enzymes which can withstand the high temperatures (over 100 °C) employed during starch gelatinisation. Indeed, at the present time only the liquefying α-amylase meets this requirement, since the saccharifying enzymes that are currently employed (a glucoamylase and a pullulanase type I) are only moderately stable (saccharification is performed at 60 °C). Therefore, in recent years a large number of new amylolytic enzymes have been isolated from thermophilic bacterial and notably archaebacterial sources (Sunna et al., 1997; Crabb & Shetty, 1999; Leveque et al., 2000).

The industrial interest in pullulanases arises from the fact that pullulanases hydrolyse the α-1,6 glucosidic linkage and, in consequence, are able to de-branch amylopectin. Therefore, when pullulanase is used in conjunction with glucoamylase for starch saccharification, glucoamylase activity is improved and the final syrup is enriched in glucose. Pullulanases that only possess α-1,6 linkage-specific activity are known as pullulanases type I (Bender & Wallenfels, 1966), whereas bifunctional pullulanases, which can also cleave α-1,4 glucosidic linkages, are known as pullulanases type II (Spreinat & Antranikian, 1990). During the last decade the number of known thermostable pullulanases (both type I and II) has increased significantly and indeed, at the present time, several extremely thermostable pullulanases are available (Suzuki et al., 1991; Mathupala & Zeikus, 1993; Rüdiger et al., 1995; Cheorl-Ho et al., 1996; Dong et al., 1997; Bertoldo et al., 1999; Duffner et al., 2000). In principle, among these enzymes, the highly thermostable pullulanases type II are the most interesting from an industrial point of view. Clearly, these enzymes could facilitate a major change in the current strategy for starch processing. First, owing to their high thermostability, such enzymes could be added before gelatinisation, at the same time as the α-amylase, and second, their bi-functionality could reduce or even obviate the glucoamylase requirement.

In previous work, we have described the isolation and preliminary characterisation of a pullulanase type II (Th-Apu) which is produced by the archaebacterium *Thermococcus hydrothermalis* AL662 (Godfroy et al., 1997; Gantelet & Duchiron, 1998; Gantelet et al., 1998). Like similar pullulanases from other archaea, Th-Apu is highly thermostable and, therefore may be useful for the starch industry (Gantelet & Duchiron, 1999). More recent genetic studies have allowed the identification and characterisation of the gene encoding Th-Apu (Erra-Pujada et al., 1999). Analysis of the primary sequence of Th-Apu has revealed that this enzyme exhibits several distinct regions and is a member of family 57 (F-57) of the glycosyl hydrolase classification system. Like the other archaeal pullulanases in this family (a putative pullulanase from *Pyrococcus abyssi* is the most closely related homologue), Th-Apu does not appear to possess the conserved primary sequence motifs which are characteristic of many other amylolytic enzymes (notably F-13 ones) (Janecek, 1997, 1998; Janecek et al., 1997; Kuriki & Imanaka, 1999). Recent biochemical analyses have been pursued on the catalytic domain (ApuΔ2), which is the only part of Th-Apu which could be successfully cloned and expressed in *Escherichia coli* (Erra-Pujada et al., 2001). This work has shown that ApuΔ2 hydrolyses both α-1,6 and α-1,4 glucosidic bonds, with the former being its preferred target. The minimal substrate for ApuΔ2 is maltotetraose, with maltotriose being the only product of pullulan hydrolysis. Furthermore, although pullulan is the preferred substrate, ApuΔ2 can hydrolyse starch, amylose and to a much lesser extent glycogen and β- and γ-cyclodextrins. In contrast, ApuΔ2 can not hydrolyse either dextran or α-cyclodextrin, the latter appearing to be an inhibitor.

Since very little biochemical data and no structural information are available for F-57 enzymes, our present work has attempted to address two of the fundamental questions concerning the F-57 pullulanases: (i) Do these enzymes possess one or two independent active sites? and (ii) Are

the F-57 pullulanases structurally related to F-13 amylolytic enzymes?

Material and methods

Enzyme production
ApuΔ2 was produced by *E. coli* cells bearing the plasmid pAPUΔ2 and was purified as previously described (Erra-Pujada et al., 2001).

Standard enzyme assay
Unless otherwise stated, activity measurements were performed using a standard reducing sugar assay (Kidby & Davidson, 1973). Usually 800 μL of substrate (1% w/v in 50 mM sodium acetate, 5 mM $CaCl_2$, pH 5.5) were pre-incubated for 10 min at 80 °C before the addition of 100 μL of ApuΔ2. Incubation at 80 °C was continued and aliquots (100 μL) were removed at regular intervals for reducing sugar analysis. One unit (UI) of ApuΔ2 activity produces 1 μmol of reducing sugars per min, under the assay conditions, using an appropriate standard.

Effects of additives and metal ions
To investigate ApuΔ2 metal ion requirement activities, calcium-free enzyme was prepared by exhaustive dialysis of ApuΔ2 solution against 50 mM sodium acetate buffer, pH 5.5 containing 50 mM EDTA, followed by dialysis against ultrapure water. Afterwards, the pullulanolytic activity of calcium-depleted ApuΔ2 was measured in the presence of various metal ions at two different concentrations (1 and 5 mM). For activity measurements in the presence of other additives such as detergents or denaturants, ApuΔ2 was pre-incubated for 1h at ambient temperature in buffer containing the additive before ApuΔ2 activity was measured. In the case of reactions involving reducing compounds, an alternative reducing sugar assay (DNS method) was employed (Bernfeld, 1955).

Influence of temperature and pH
To investigate the thermoactivity of ApuΔ2 in the presence of pullulan, amylose or pNP-maltotriose, the standard or colorimetric (for pNP-maltotriose) assays were performed at various temperatures (30 to 120 °C) in the presence (5 mM) or absence of Ca^{2+} ions. For the latter, calcium-depleted ApuΔ2 was used (see above). The colorimetric assay was a standard pNP method. 160 μL of the buffered substrate solution (10 mM pNP-maltotriose in 50mM sodium acetate) was incubated for 10 min at 80 °C before adding 20 μL of ApuΔ2. Afterwards, aliquots of the reaction mixture (25 μL) were removed at regular intervals for analysis. pNP release during hydrolysis was quantified by monitoring the absorbance at 401 nm of each sample after the addition of 975 μL of Na_2CO_3(50 mM). One unit (UI) of amylolytic activity was defined as the quantity of enzyme necessary to release 1 μmol of pNP per min under the assay conditions, using pNP as the standard.

For thermostability measurements, ApuΔ2 or calcium-depleted ApuΔ2 were incubated at different temperatures for periods of time up to 600 minutes. At regular intervals aliquots of enzyme were removed and pullulanolytic activity was measured using the standard assay.

For pH dependence, activity measurements were performed using both pullulan and amylose as substrates in the standard assay, using Britton and Robinson type universal buffers (pH range 3.5 to 12) (McKenzie, 1969) in place of sodium acetate buffer.

Acarbose inhibition
The effect of acarbose on both pullulan and amylose hydrolyses was determined using the standard assay in the presence of various concentrations of acarbose (50 to 250 mM for pullulanolytic activity and 500 μM and 1 mM for amylolytic activity). Kinetic data analysis was performed using SigmaPlot 2000 version 6.10 (SPSS Inc., Chicago, USA) equipped with the Enzyme kinetics module.

Protein secondary structure analysis
Far UV CD and RAMAN spectral data were collected for solutions of ApuΔ2 in ultrapure water using a Jasco J-810 spectropolarimeter and a Jobin-Yvon S-3000 RAMAN spectrometer respectively. Prediction of secondary structure was performed using the GOR III (Gibrat et al., 1987) and the Chou-Fasman (using the CF67 set – (Alix, 1999)) routines. Computer-generated predictions were adjusted in order to reflect the experimentally-derived global secondary structure contributions using the LINK procedure (Alix, 1995, 1997). Finally after comparison of the adjusted prediction data, a consensus prediction was defined.

Results and discussion

Effect of various compounds and metal ions on ApuΔ2 activity
The addition of various compounds to ApuΔ2-catalysed hydrolyses of pullulan produced extremely variable results depending on the exact nature of the compound although clear tendencies could be observed (Tab. 1). The reducing compounds (DTT and 2-mercaptoethanol) appeared to favour activity. This is rather surprising since ApuΔ2 does not contain any disulphide bond-forming cysteine residues, but a similar effect was observed in the case of the pullulanase type II from *Pyrococcus woesei* (Rüdiger et al., 1995). Predictably the protein denaturants, urea and guanidinium hydrochloride, led to decreased activity, with the latter compound inducing the most dramatic effect. This may suggest that ionic interactions are critical for the extreme stability of ApuΔ2 (Smith & Scholtz, 1996; Gokhale et al., 1999; Makhatadze, 1999). In keeping with this notion, ApuΔ2, like other F-57 pullulanases (Rüdiger et al., 1995; Dong et al., 1997), was stimulated by the presence of detergents, although

Table 1. Activity of ApuΔ2 in the presence of various compounds.

Compound	Concentration	Residual activity (%)
EDTA	1 mM	29
	10 mM	13
SDS	1 mM	121
	5 mM	1
Triton X-100	0.5 % (v/v)	150
	1% (v/v)	146
Urea	1 M	99
	5 M	46
Guanidinium-HCl	0.25 M	75
	0.5 M	3
	5 M	0
N-bromosuccinimide	56 mM	0
Dithiothreitol	10 mM	93
	50 mM	154
2-Mercaptoethanol	10 mM	103
	100 mM	111

Table 2. Influence of metal ions on ApuΔ2 activity.

Metal ion	Residual activity (%)	
	1 mM	5 mM
Ca^{2+}	257	248
Mg^{2+}	118	135
Na^{+}	96	120
Mn^{2+}	76	73
Co^{2+}	65	17
Ni^{2+}	39	32
Zn^{2+}	0	0
Cu^{2+}	0	0
Fe^{2+}	0	0

the ionic detergent, SDS, was only tolerated at low concentrations. Finally, like the recently described *Desulforococcus* enzyme (Duffner et al., 2000), but unlike a *Pyrococcus* one (Rüdiger et al., 1995), strong inhibition of ApuΔ2 activity was achieved using low concentrations of N-bromosuccinimide.

With regard to metal ions (Tab. 2), only Ca^{2+} and to a lesser extent Mg^{2+} and Na^{+} were capable of restoring the activity of calcium depleted-ApuΔ2. This observation, which is similar to previous results for a pullulanase type I (Kim et al., 1996), reveal an important difference between ApuΔ2 and the other archaeal F-57 pullulanases.

Measurement of temperature influence

Thermostability measurements performed both in the presence and absence of calcium ions or substrate (Figs 1A,B) revealed that calcium concentrations in excess of the minimal calcium requirement (0.09 mM) (Erra-Pujada et al., 2001) confer a stabilising effect on ApuΔ2, allowing it to remain very active during prolonged periods of

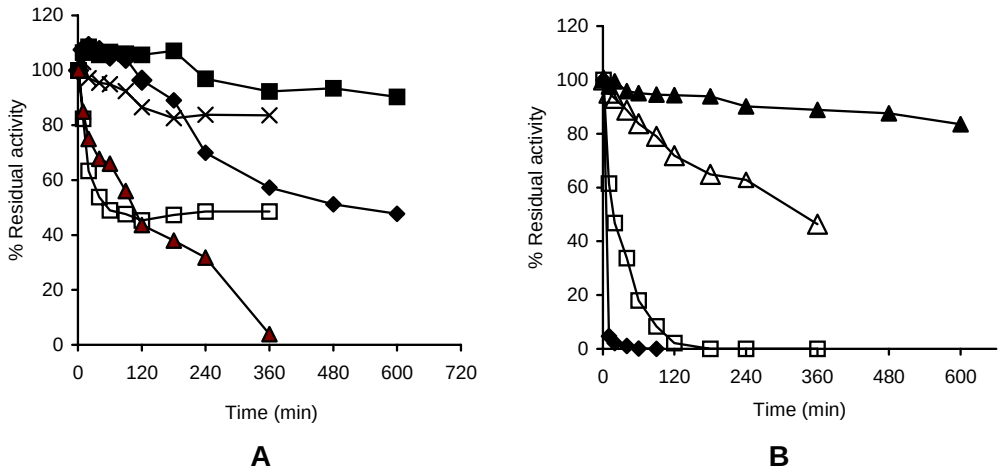

Fig. 1. The effect of substrate (**A**) or calcium ions (**B**) on the thermostability of ApuΔ2. In panel **A**, ♦ is 80 °C and ■ is 80 °C + pullulan (0.5% w/v); □ is 85 °C and × is 85 °C + pullulan (0.5% w/v); ▲ is 90 °C + pullulan (0.5% w/v). In panel **B**, ♦ is 90 °C and ▲ is 90 °C + calcium (5 mM); △ is 100 °C + calcium (5 mM); □ is 110 °C + calcium (5 mM)

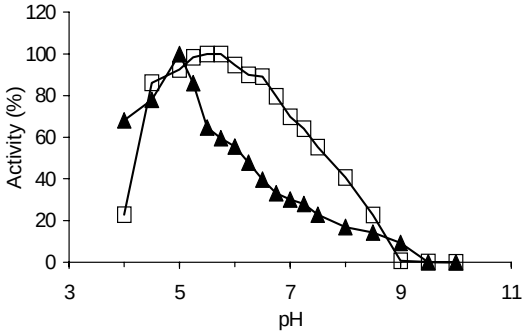

Fig. 2. pH dependence of ApuΔ2 activity in the presence of pullulan (□) or amylose (▲) as substrates.

time at 90 °C. Thermoactivity measurements in the presence of pullulan or amylose revealed that ApuΔ2 displays maximal activity at a similar temperature (80 °C) and this thermoactivity can be increased to 105 °C by the addition of calcium. In contrast, the presence of pNP-maltotriose was more stabilising than the polysaccharides. Indeed, this substrate increased the temperature at which ApuΔ2 activity was maximal to 115 °C. Interestingly, a similar stabilisation of a pullulanase from *Bacillus acidopulllulyticus* has been previously reported (Kusano et al., 1990).

pH dependence

When the pH dependence of ApuΔ2 activity was measured (Fig. 2) in the presence of pullulan, a typical bell shaped graph was generated which revealed that ApuΔ2 activity was stable over a wide pH range (4.5 to 6.5). This pH dependence is broader than that reported for the *P. woesei* pullulanase (Rüdiger et al., 1995), but almost identical to that reported for the *P. furiosus* one. In contrast, when amylose was used as substrate, the pH dependence of ApuΔ2 activity was described by a reproducibly different graph which showed that maximal activity was obtained in a very narrow range around pH 5.0. This result suggests a difference in the pullulanolytic and amylolytic activities of ApuΔ2 which, at the present time, remains difficult to explain.

Inhibitor competition experiments

The pseudotetrasaccharide, acarbose, which is known to be a potent inhibitor against many F-13 and F-15 enzymes (Wilcox & Whitaker, 1984; Svensson & Sierks, 1992), was added to ApuΔ2-catalysed reactions. Hydrolyses using either pullulan or amylose as substrates were inhibited. Kinetic analysis of this inhibition revealed that acarbose is a good inhibitor of pullulanase activity ($K_i = 25 \pm 5.3$ μM), whereas it is a poor inhibitor of amylolytic activity ($K_i = 1063 \pm 73$ μM). In order to identify the type of inhibition in each case, Woolf-Augustinsson-Hofstee plots were generated (Figs 3A,B). Clearly, in the case of pullulan hydrolysis V_{max} remains constant while the apparent K_m value increases with inhibitor concentration. This behaviour is typical of competitive inhibition. In contrast, the addition of acarbose to ApuΔ2-catalysed amylose hydrolyses leads to the opposite behaviour (i.e. decreased V_{max} accompanied by a constant K_m value) which is indicative of non-competitive inhibition (Cornish-Bowden, 1995). In the absence of detailed structural data, a clear interpretation of these results is

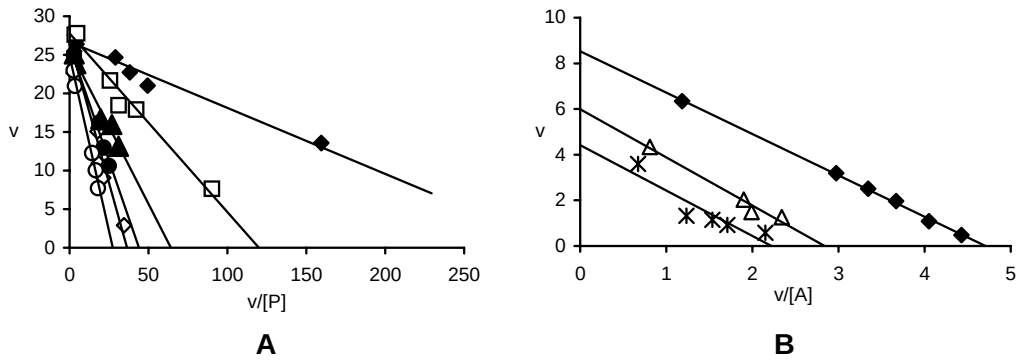

Fig. 3. Woolf-Augustinsson-Hofstee plots for acarbose inhibition of ApuΔ2 in the presence of pullulan (panel **A**) and amylose (panel **B**). Each graph is composed of curves for different acarbose concentrations. In panel **A**, these concentrations are represented as follows: ♦ none; □ 50 μM; ▲ 100 μM; ● 150 μM; ◇ 200 μM; ○ 250 μM. In panel **B**, acarbose concentrations are: ♦ none; △ 500 μM; ∗ 1000 μM.

Table 3. Spectroscopic analysis of global contributions of secondary structural elements in ApuΔ2.

Method	α-Helical structure		β-Strand structure		Undetermined structure	
	AA[a]	% ApuΔ2	AA	% ApuΔ2	AA	% ApuΔ2
RAMAN	300	39	322	42	147	19
CD	300	39	415	53.9	54	7.1

[a] AA is estimated number of amino acids involved in a given configuration.

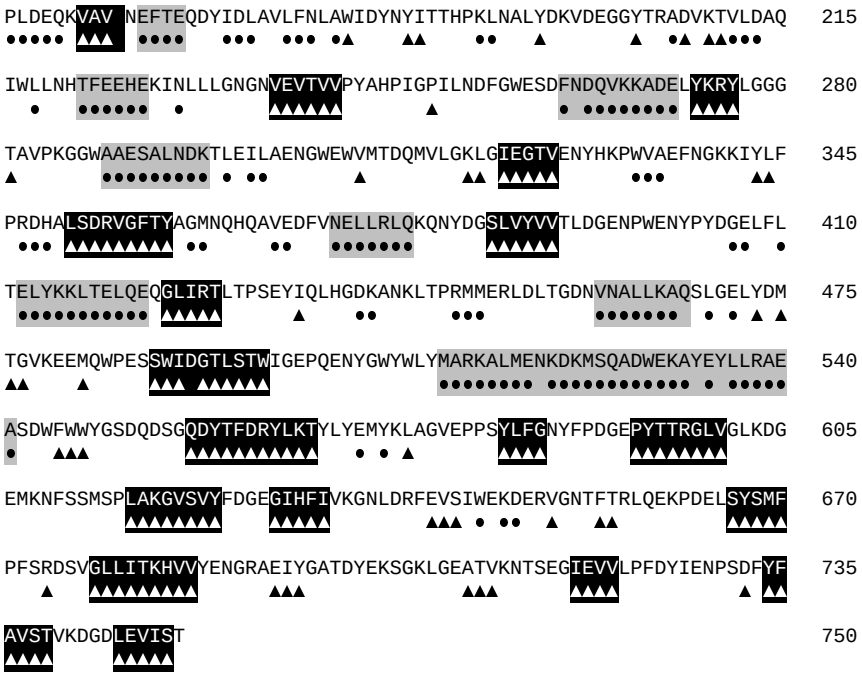

Fig. 4. The consensus results for experimentally-adjusted predictions of the secondary structure of a part of ApuΔ2, corresponding to amino acids 151 to 750 (corresponding to regions 3 and 4 in the text). The symbols ▲ and ● represent β-strand and α-helical conformations respectively. The shaded areas indicate the probable locations of α-helices (grey shading) and β-strands (white lettering on a black background).

impossible. However, taken together with the pH dependence data, these data point to the existence of two independent active sites within ApuΔ2. In this case, the fact that N-bromosuccinimide simultaneously abolishes both activities would suggest that both sites are equally affected, whereas this reagent has been found to have a discriminatory effect on the hydrolytic activities of other pullulanases type II (Ara et al., 1995; Rüdiger et al., 1995). In addition, it is interesting to note that a F-13 pullulanase type II from *Thermoanaerobacter thermohydrosulfuricum*, was not inhibited by acarbose (Saha et al., 1988). This suggests that the active sites of this enzyme and that of ApuΔ2 are not structurally related.

Secondary structure analyses

The measurement of high quality UV circular dichroism and RAMAN spectra, allowed the accurate estimation of contributions of secondary structural elements to the global structure of ApuΔ2 (Tab. 3). Overall, the data derived from these two techniques were similar and indicated that approximately 39% of the ApuΔ2 secondary structure is composed of α-helices. These experimentally-determined values were then used to adjust the results of prediction analyses, which had been performed using the Chou and Fasman (CF67 set) (Chou & Fasman, 1978; Alix, 1999) or GOR III methods (Gibrat et al., 1987). In order to perform this adjustment, the previously

described LINK procedure (ALIX, 1995; DEBELLE et al., 1998) was employed. This step allowed four adjusted predictions (CD- and RAMAN-adjusted data for each prediction method) to be generated. The good agreement between these adjusted predictions facilitated the definition of a final consensus prediction which revealed that ApuΔ2 is composed of four distinct regions (Fig. 4). An N-terminal region (a.a. 1 to 32) composed of β-strands is followed by a second region (a.a. 33 to 126) of undetermined structure, which precedes a third major region (a.a. 127 to 578) of alternating β-strands and α-helices. Finally, one can distinguish a C-terminal region (a.a. 579-769) which is composed of a succession of β-strands. Interestingly, the secondary structure arrangement of these latter two regions is reminiscent of that of domains A and C of the F-13 α-amylases, i.e. the alternating β-strand/α-helix pattern may be the $(\alpha/\beta)_8$-fold and the C-terminal region, composed exclusively of β-strands, may constitute the Greek key motif (MATSUURA et al., 1980, 1984; BUISSON et al., 1987; SVENSSON, 1994; JANECEK, 1997).

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