

Article

A New Esterase from *Thermobifida halotolerans* Hydrolyses Polyethylene Terephthalate (PET) and Polylactic Acid (PLA)

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Abstract: A new esterase from *Thermobifida halotolerans* (Thh_Est) was cloned and expressed in *E. coli* and investigated for surface hydrolysis of polylactic acid (PLA) and polyethylene terephthalate (PET). Thh_Est is a member of the serine hydrolases superfamily containing the -GxSxG- motif with 85–87% homology to an esterase from *T. alba*, to an acetylxylylan esterase from *T. fusca* and to various *Thermobifida* cutinases. Thh_Est hydrolyzed the PET model substrate bis(benzoyloxyethyl)terephthalate and PET releasing terephthalic acid and mono-(2-hydroxyethyl) terephthalate in comparable amounts (19.8 and 21.5 mmol/mol of enzyme) while no higher oligomers like bis-(2-hydroxyethyl) terephthalate were detected. Similarly, PLA was hydrolyzed as indicated by the release of lactic acid. Enzymatic surface hydrolysis of PET and PLA led to a strong hydrophilicity increase, as quantified with a WCA decrease from 90.8° and 75.5° to 50.4° and to a complete spread of the water drop on the surface, respectively.

Keywords: enzymatic polyester functionalization; *Thermobifida halotolerans* esterase

1. Introduction

Enzymes have been used for a long time in many industrial processes [1–4] and consumer applications [5–7]. They allow simplification of complex chemical processes [8], synthesis of new molecules [9], or as additives they can increase the performance of the product in which they are formulated [10,11]. One of the fields in which enzymes are increasingly capturing the interest of researchers across the globe is material science. There are several interesting examples describing the functionalization of natural fibers using enzymes in order to increase antibacterial properties [12], antioxidant capacity [12–14], or to increase compatibility with synthetic materials [15]. Nevertheless, it is still a challenge to find suitable enzymes for the biotransformation of non-natural and polymeric substrates.

Recently, enzymes have been demonstrated to be suitable alternative tools for hydrophilization of polymer surfaces. Hydrophilization of polyesters is essential for numerous applications of PLA and PET, ranging from textiles to composites and medical devices [1,16]. Typically, chemical methods [17,18] or plasma technology are used to increase the hydrophilicity of polymers [19,20] or to create reactive groups on the surface of inert materials [21]. Enzyme-based approaches would avoid the use of harsh chemicals [22] and/or require considerably less energy [23].

Poly(alkyleneterephthalates) (PAT), and particularly polyethylene terephthalate (PET), are one of the most widely used synthetic polymers worldwide [24] which can be functionalized via limited enzymatic hydrolysis of the ester bond of the polymer backbone [25–29]. Cutinases [30–32], lipases [29,32], esterases [26,30,33] including paranitrobenzyl esterase [34] have been assessed for this purpose.

Among cutinases, representatives from *Aspergillus oryzae*, *Humicola insolens* [35], *Penicillium citrinum* [36], *Fusarium solani* [30,37], *Thermobifida fusca* [29] and *Thermobifida cellulolysitica* [27] have been described to hydrolyze PET. Considerably fewer lipases have been described to hydrolyze PET, including enzymes from *Candida antarctica* [25], *Thermomyces lanuginosus* [32], *Burkholderia spp.* [38] and *Triticum aestivum* [39]. Esterases were believed to have less potential for surface hydrolysis of polyesters than cutinases or lipases [26,34].

Here we demonstrate for the first time that the esterase Thh_Est from *Thermobifida halotolerans* can efficiently hydrolyze PET and PLA thereby considerably increasing surface hydrophilicity of these materials.

2. Materials and Methods

2.1. Chemicals

Methanol and acetonitrile were purchased from Roth (Carl Roth, Karlsruhe, Germany). All other chemicals were of analytical grade from SIGMA (Germany) except the model substrate

nucleotide sequence of *Thh_est* has been deposited in the GenBank database under submission JQ339742.

2.6. Expression and Purification

Thh_Est was expressed in shake flasks. Therefore the plasmid was freshly transformed in *E. coli* BL21-Gold(DE3). Transformants were used to inoculate 20 mL Luria-Bertani (LB) broth supplemented with 40 µg/mL kanamycin. The culture was grown overnight on a rotary shaker at 30 °C and 160 rpm. For inoculation of the main culture, 1 mL of the overnight culture was transferred to 200 mL of the same media and incubated at 30 °C. At an optical density (600 nm) between 0.6 and 0.8 the culture was cooled down to 20 °C and induced by addition of 0.05 mM IPTG. After induction for 24 h the cells were harvested by centrifugation (3,200 g, 4 °C, 20 min). Cell pellet was disrupted by sonification using three-times 30-s pulses under ice cooling (cell disruptor from BRANSON Ultrasonics). Purification of the enzyme was performed by affinity chromatography according to the manufacturer's instructions (IBA BioTAGnology, Germany). Afterwards the HisTag elution buffer was exchanged with 100 mM Tris HCl pH 7.0 by the use of PD-10 desalting columns (Amersham Biosciences).

2.7. Esterase Activity Assay

Esterase activity using *p*-nitrophenyl acetate (PNPA) and *p*-nitrophenyl butyrate (PNPB) as substrates was measured at 25 °C. For the final assay mixture, 200 µL of the substrate solution was diluted in 50 mM Tris HCl buffer pH = 7 and mixed with 20 µL enzyme solution. Activity was determined by measuring the increase of the absorbance at 405 nm, which indicated an increase of *p*-nitrophenol ($\epsilon_{405\text{nm}} = 11.86 \text{ mmol}^{-1}\text{cm}^{-1}$) due to hydrolysis of PNPA or PNPB. A Spectromax Plus 384 plate reader (Molecular Devices) was used to follow the reaction. The activity was calculated in units, where 1 unit had been defined as being the amount of enzyme required to hydrolyze 1 µmol of substrate per minute under the given assay condition. A blank was measured using 20 µL buffer instead of sample.

2.8. PET and PLA Hydrolysis

Prior to the enzyme treatment, PET and PLA films were cut into pieces of 10 × 20 mm and washed in a three consecutive steps. In a first step, each PET piece was washed with a solution of Triton-X100, in a second step 100 mM Na₂CO₃ and finally deionized water was used. Each washing step lasted 30 min and was performed at 50 °C for PET and 37 °C for PLA. The final enzyme concentration was 100 mg/mL diluted in tris buffer (100 mM) pH = 7. Incubations were made at 130 rpm and 50 °C for PET and 37 °C for PLA. Hydrolysis products were measured by using high performance liquid chromatography - (HPLC) as described below. Controls were performed with heat-inactivated enzyme.

2.9. Hydrolysis of PET Model Substrate bis(benzoyloxyethyl)terephthalate

Hydrolysis of the PET model substrate bis(benzoyloxyethyl)terephthalate (3PET) was performed in 2 mL Eppendorf tubes as previously described [27]. 10 mg 3PET were incubated in 1000 µL of 100 mM

K_2HPO_4/KH_2PO_4 containing 100 $\mu\text{g/mL}$ enzyme at 50 °C and pH 7.0 for 2 hours and 350 rpm. The released products were analyzed by using HPLC as described below.

2.10. Analysis of PET, 3PET and PLA Hydrolysis Released Products

PET and 3PET soluble released products were analyzed as previously described by Eberl *et al.* [32]. After enzymatic treatment proteins were precipitated using 1:1 (v/v) methanol abs. on ice. Samples were centrifuged (Hettich MIKRO 200 R, Tuttlingen, Germany) at 16,000 $\times g$ at 0 °C for 15 min. The supernatant for measurement was brought to an HPLC vial and acidified by adding 1 μL of HCl concentrated. The HPLC used was a DIONEX P-580 PUMP (Dionex Cooperation, Sunnyvale, USA) equipped with an ASI-100 automated sample injector and a PDA-100 photodiode array detector. Analysis of TA, benzoic acid (BA), 2-hydroxyethyl benzoate (HEB), mono-(2-hydroxyethyl) terephthalate (MHET) and bis-(2-hydroxyethyl) terephthalate (BHET) a reversed phase column RP-C18 (Discovery® HS-C18, 5 μm , 150 \times 4.6 mm with precolumn, Supelco, Bellefonte, USA) was used.

Lactic Acid (LA) was also determined using the same conditions. Analysis was carried out with 20% acetonitrile, 20% 10 mM sulfuric acid and 60% (v/v) water as eluent. The flow rate was set to 1 mL min⁻¹ and the column was maintained at a temperature of 25 °C. The injection volume was 10 μL . Detection of the analytes was performed with a photodiode array detector at the wavelength of 241 nm.

2.11. PET and PLA Hydrophilicity Measurements

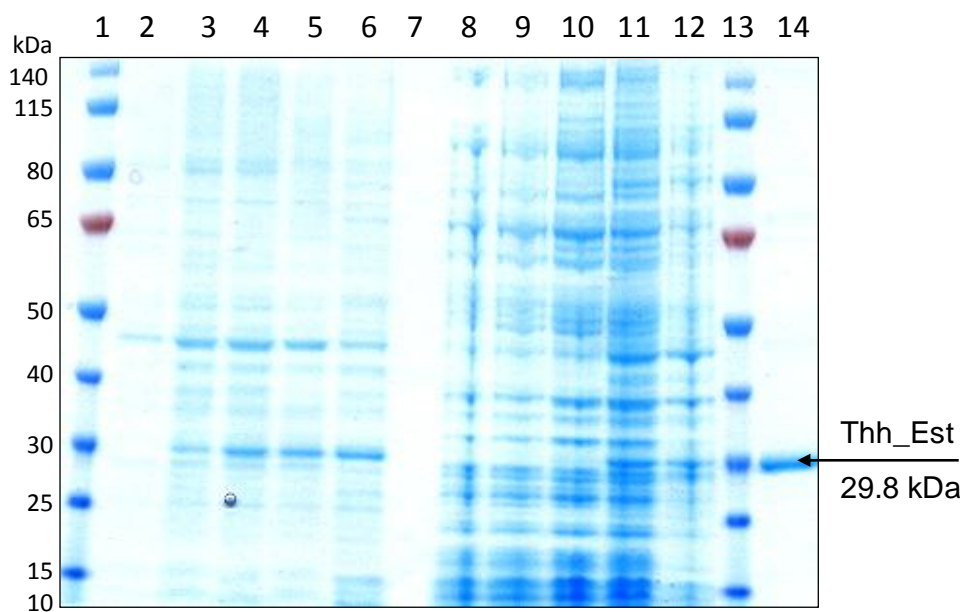
Water contact angles of the PET and PLA films after enzymatic treatment were measured using a Contact Angle and Surface Tension Analyzer (Kruss GmbH, Hamburg, Germany). Deionized water was used as test liquid with a drop size of 3 μL and the speed of 300 $\mu\text{L/s}$. Contact angles were measured after 3 s and data are obtained from the averages of the measurements taken from at least 5 different points of the sample surface.

3. Results and Discussion

3.1. Cloning, Sequence Analysis and Heterologous Expression in *E. coli* of an Esterase from *Thermobifida Halotolerans*

Cutinases from *Thermobifida fusca*, *T. cellulositytica* and *T. alba* have shown to hydrolyze PET-films [27,42]. However, for industrial processes, more efficient enzymes are still needed while their potential to surface hydrophylyze PLA has been largely neglected. In 2008, *Thermobifida halotolerans* has been isolated from a salt mine sample collected from Yunnan province (Southwest China, [43]) and classified as a novel species of *Thermobifida*. In order to identify homologous cutinases from this novel species, primers were designed, based on the genome sequences of cutinases from *T. fusca* YX. By the use of the designed primers, the gene coding for Thh_Est was amplified from the genomic DNA of *T. halotolerans*. The open reading frame of the gene (789 bp) encodes for a protein comprising 262 amino acids and a calculated mass of 28.7 kDa. The gene comprised a GC content of 68% which is expected for the genus *Thermobifida*. Sequence analysis of Thh_Est

Figure 2. SDS-PAGE analysis of Thh_Est expressed in *E. coli* BL21-Gold (DE3). Samples were withdrawn at different times of induction, centrifuged, disrupted and centrifuged again. Lane 1 and 13: PageRuler Prestained Protein Ladder (Fermentas); lane 2-6: cleared lysates from induced cells after 0, 3, 6 and 10 h of induction with 0.05 mM IPTG; lane 7: empty; lane 8-12: insoluble cell fractions from induced cells after 0, 3, 6 and 10 h of induction; lane 14: purified Thh_Est.

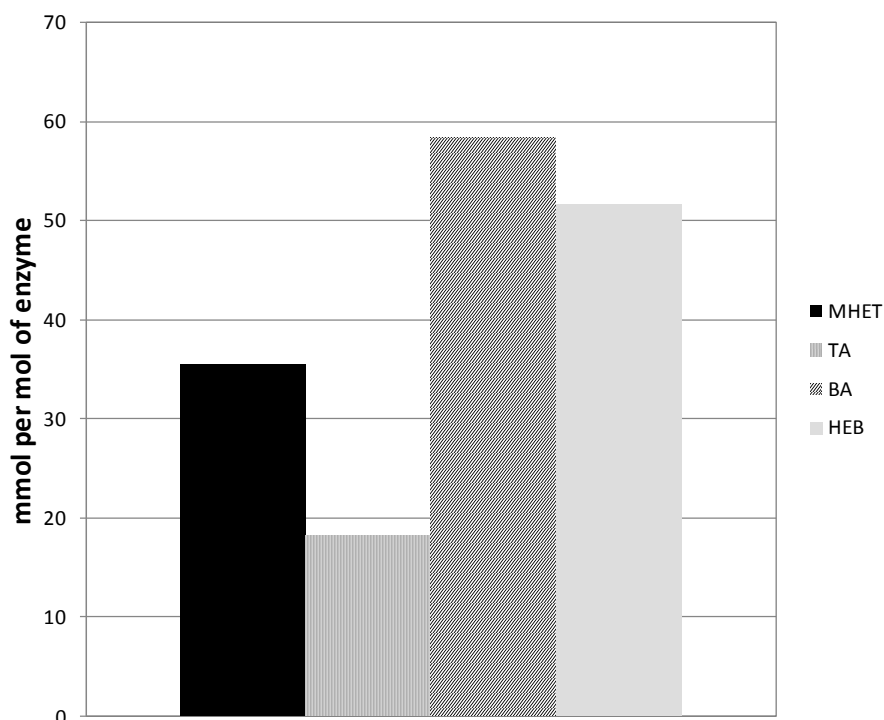


3.2. Hydrolysis of PLA Films

The ability of the Thh_Est to hydrolyze PLA films was assessed by analyzing the released products via HPLC-UV after hydrolysis and measurements of the hydrophilicity increase as WCA. The only released product found in the incubation solution was lactic acid in a concentration of 14.5 μM while no higher oligomers were detected. The degree of hydrophilization achieved was significant since it was not possible to measure a final value of the WCA due to the fact that the water drop was a complete spread on the polymer surface.

3.3. Hydrolysis of the PET Model Substrate bis(benzoyloxyethyl)terephthalate

Hydrolysis of the model substrate bis(benzoyloxyethyl)terephthalate (3PET) has been proven first to be valuable for the screening of enzymes capable of PET hydrolysis [40] and to provide important mechanistic information [27,32,34,42] about the type of hydrolysis mechanism. In the case of the new esterase Thh_Est, the hydrolysis product found in highest concentration was BA, followed by HEB, both in a comparable concentration higher than 50 mmol per mol of enzyme. In contrast, MHET and TA were detected in a lower amount, whereas no BHET was observed (Figure 3).

Figure 3. Hydrolysis of the PET model substrate 3PET with the esterase Thh_Est.

The 3PET hydrolysis product pattern of Thh_Est was similar to the profile previously reported for the cutinase 2 from *Thermobifida cellulositilytica* (Thc_Cut2). Other *Thermobifida fusca* cutinases [27,32] likewise released comparable amounts of BA and HEB whereas the ratio between MHET and TA was higher for Thh_Est. This might indicate a more exo-type hydrolysis mechanism of the Thh_Est esterase when compared to *Thermobifida* cutinases. In contrast, a *Humicola insolens* (Hi_Cut) cutinase released the terminal BA and MHET as major hydrolysis products [42]. A very similar hydrolysis profile was found for the *para*-nitrobenzylesterase from *Bacillus subtilis* (BsEstB) [34], although in this case the amount of release products was much lower than for Thh_Est or Hi_Cut. Interestingly, the overall amount of released products per mol of Thh_Est was significantly higher than the values reported for cutinases from *Thermobifida* species Thc_Cu1, Thc_Cut2 and Thf_42 [27].

3.4. Hydrolysis of PET Films

Upon incubation with PET, the new esterase from *T. halotolerans* only released MHET and TA while no BHET was detected. The amount of MHET and TA released were comparable with around 19.8 and 21.5 mmol/mol of enzyme, respectively. Previously, for a lipase from *T. lanuginosus* [29] a ten times higher release of MHET than TA from amorphous fibers and 3 times more from semi-crystalline PET fibers was reported. Interestingly, only in the case of amorphous fibers was BHET detected. Similarly, Eberl *et al.* reported higher amounts of MHET than of TA when the same lipase was used [32]. In the case of cutinase from *F. solani* again higher MHET to TA ratios were found and BHET was detected in non-significant amounts [25]. For cutinases from *Fusca sp.* comparable amounts of MHET and TA released from PET were reported, with no detection of BHET, when PET fabrics of around 40% crystallinity were used as substrate [32]. On the other hand,

cutinases from *Thermobifida fusca* DSM 44342 and cutinase 1 from *Thermobifida cellulolysitca* showed an opposite trend with higher amounts of TA than MHET. However, prolonged incubation together with the ability of the both enzymes to endo-wise hydrolyze the polyester may lead to this view [27].

3.5. Hydrophilicity Measurement of Enzymatically Treated PLA and PET

Thh_Est was able to hydrolyze films of both PLA and PET (Table 2). Provided a complete removal of the enzyme adhered in the polymer surface it is possible to compare the degree of hydrophilicity achieved by Water Contact Angle (WCA) measurement [29]. In the case of PLA films the hydrophilicity increase achieved was significantly higher than obtained for PET. The WCA in the case of PLA decreased from 75.5 ° to a complete spread of the water drop on the polymer surface after deposition. For PET it was possible to decrease the WCA from 90.8 ° to 50.4 °.

Table 2. Hydrophilicity increase (quantified as Water contact angle WCA decrease) of PET and PLA upon surface hydrolysis with the esterase Thh_Est.

	Initial	Control	Enzyme treated
PET	90.8 ° ± 3.3 °	74.8 ° ± 2.3 °	50.4 ° ± 9.9 °
PLA	75.5 ° ± 2.7 °	68.4 ° ± 2.3 °	<20 °

Enzymatic hydrophilization of polyester surfaces has applications [1] ranging from improved coating with PVC [46] to more efficient textile finishing processes [29]. The advantage of enzymes derived from *Thermobifida species* is high thermal stability which allows working at temperatures below but close to the glass transition temperature of the polymer. These working conditions facilitate a high mobility of the polymer chains leading to higher accessibility of the polymer for enzymatic attack.

Concluding the results of this paper, the new esterase *Thermobifida* Thh_Est has a potential for both PET and PLA surface hydrolysis, similar to cutinases from the same genus. This is rather unexpected since cutinases were previously believed to be superior to esterases in terms of polyester hydrolysis [26,34].

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Appendix

Alignment of the primary sequence of Thh_Est, esterase from *T. alba* (Tha_Est119, Genbank Genbank BAK48590.1), cutinases from *T. cellulositytica* (Thc_Cut1 and Thc_Cut2, Genbank ADV92526 and ADV92527), cutinase 1 from *T. alba* (Tha_Cut1, Genbank ADV92526) and acetylxylan esterase from *T. fusca* (TFAXE, Genbank ADM47605.1). Red: amino acids of the catalytic triade. Bold: conserved GxSxG motif.

	10	20	30	40	50
Tha_Est119	MANPYERGPN	PTESMLEARS	GPFSVSEERA	SFRGADGFGG	GTIYYPRENN	
TFAXE	MANPYERGPN	PTDALLEARS	GPFSVSEENV	SRLSASGFGG	GTIYYPRESN	
Tha_Cut1	MANPYERGPN	PTDALLEASS	GPFSVSEENV	SRLSASGFGG	GTIYYPRENN	
Thc_Cut1	MANPYERGPN	PTDALLEASS	GPFSVSEENV	SRLSASGFGG	GTIYYPRENN	
Thc_Cut2	MANPYERGPN	PTDALLEARS	GPFSVSEERA	SFRGADGFGG	GTIYYPRENN	
Thh_Est	MANPYERGPN	PTNSSIEALR	GPFRVDEERV	SRLQARGFGG	GTIYYPTDNN	
	60	70	80	90	100
Tha_Est119	TYGAVAIISP	YTGTQSSIAW	LGERIASHGF	VVIAIDTNTT	LDQPDSRARQ	
TFAXE	TYGAVAIISP	YTGTQSSIAW	LGERIASHGF	VVITIDTITT	LDQPDSRAEQ	
Tha_Cut1	TYGAVAIISP	YTGTQSSIAW	LGGRIASHGF	VVITIDTITT	LDQPDSRAEQ	
Thc_Cut1	TYGAVAIISP	YTGTQSSIAW	LGERIASHGF	VVITIDTITT	LDQPDSRAEQ	
Thc_Cut2	TYGAVAIISP	YTGTQASVAW	LGERIASHGF	VVITIDTNTT	LDQPDSRARQ	
Thh_Est	TFGAVAIISP	YTGTQSSISW	LGERLASHGF	VVMTIDTNTT	LDQPDSRASQ	
	110	120	130	140	150
Tha_Est119	LNAALDYMLT	DASSAVRNRI	DASRLAVMGH	SMGGGGTLRL	ASQRPDLKAA	
TFAXE	LNAALNHMIN	RASSTVRSRI	DSSRLAVMGH	SMGGGGTLRL	ASQRPDLKAA	
Tha_Cut1	LNAALNHMIN	RASSTVRSRI	DSSRLAVMGH	SMGGGGTPRL	ASQRPDLKAA	
Thc_Cut1	LNAALNHMIN	RASSTVRSRI	DSSRLAVMGH	SMGGGGTLRL	ASQRPDLKAA	
Thc_Cut2	LNAALDYMIN	DASSAVRSRI	DSSRLAVMGH	SMGGGGTLRL	ASQRPDLKAA	
Thh_Est	LDAALDYMVE	DSSYSVRNRI	DSSRLAAMGH	SMGGGGTLRL	AERRPDLQAA	
	160	170	180	190	200
Tha_Est119	IPLTPWHLNK	SWRDITVPTL	IIGAEDTIA	SVTLHSKPFY	NSIPSPDCKA	
TFAXE	IPLTPWHLNK	NWSSVTVPTL	IIGADLDTIA	PVATHAKPFY	NSLPSSISKA	
Tha_Cut1	IPLTPWHLNK	NRSSVTVPTL	IIGADLDTIA	PVATHAKPFY	NSLPSSISKA	
Thc_Cut1	IPLTPWHLNK	NWSSVTVPTL	IIGADLDTIA	PVATHAKPFY	NSLPSSISKA	
Thc_Cut2	IPLTPWHLNK	NWSSVRVPTL	IIGADLDTIA	PVLTHARPFY	NSLPTSISKA	
Thh_Est	IPLTPWHTDK	TWGSVRVPTL	IIGAENDTIA	SVRSHSEPFY	NSLPGSLDKA	
	210	220	230	240	250
Tha_Est119	YLELDGASHF	APNITNKTIG	MYSVAWLKRF	VDEDTRYTQF	LCPGPRTGLL	
TFAXE	YLELDGATHF	APNIPNKIIG	KYSVAWLKWF	VDNDRYTQF	LCPGPRDGLF	
Tha_Cut1	YLELDGATHF	APNIPNKIIG	KYSVAWLKRF	VDNDRYTQF	LCPGPRDGLF	
Thc_Cut1	YLELDGATHF	APNIPNKIIG	KYSVAWLKRF	VDNDRYTQF	LCPGPRDGLF	
Thc_Cut2	YLELDGATHF	APNIPNKIIG	KYSVAWLKRF	VDNDRYTQF	LCPGPRDGLF	
Thh_Est	YLELDGASHF	APNLSNTTIA	KYSISWLKRF	VDDDRYTQF	LCPGPSTGWG	
	260				
Tha_Est119	SDVEEYRSTC	PF				
TFAXE	GEVEEYRSTC	PF				
Tha_Cut1	GEVEEYCSTC	PF				
Thc_Cut1	GEVEEYRSTC	PF				
Thc_Cut2	GEVEEYRSTC	PF				
Thh_Est	SDVEEYRSTC	PF				