Modification of catalytically important carboxy residues in endoglucanase D from *Clostridium thermocellum*

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Endoglucanase D (EC 3.2.1.4; EGD) from *Clostridium thermocellum* is rapidly ($k = 216 \text{ M}^{-1} \cdot \text{min}^{-1}$) and almost completely (> 95%) inactivated with Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3'-sulphonate). Spectrophotometric analysis at 340 nm reveals that eight carboxy residues react, whereas specific ligands protect one residue against modification. The enzyme retains its full activity under the latter conditions. The kinetics and pH-dependence of inactivation point towards the involvement of one or more essential carboxy groups with a pK_a of 5.7–5.8. Samples modified in the absence or presence of ligand were analysed by reversed-phase liquid chromatography after proteolysis with subtilisin. Dual-wavelength monitoring at 214 and 340 nm during this fractionation leads to the identification of a putatively active-site peptide (Gly-508–Ala-562) which was further characterized by amino acid and partial *N*-terminal sequence analyses. Asp-546 and Glu-555 are postulated as possible active-site residues. This follows from alignments using ten endoglucanase sequences belonging to the same family. Strong local conservation suggests that this C-terminal sequence is structurally and/or functionally important.

INTRODUCTION

More than 50 genes from various organisms (fungi, bacteria and plants) involved in cellulose and hemicellulose hydrolysis have been cloned and sequenced (Béguin, 1990). Comparison of the primary structures of endoglucanases (EC 3.2.1.4) and cellobiohydrolases (EC 3.2.1.91) by hydrophobic-cluster analysis has led to a classification of these enzymes into six distinct families, A–F, and locally conserved, catalytically and/or structurally important residues can be identified (Henrissat *et al.*, 1989). Their function can be examined by specific chemical modification and site-directed mutagenesis. This is exemplified for an endocellulase from family A (Py *et al.*, 1991) and for two enzymes from family C (Tomme & Claeysens, 1989; Mitsuishi *et al.*, 1990).

Endoglucanase D (EGD), one of the seven characterized endoglucanases from the Gram-positive, anaerobic bacterium *Clostridium thermocellum*, is easily purified from a hyperproducing *Escherichia coli* clone and crystallizes readily (Joliff *et al.*, 1986b,c). For this representative member of cellulase family E, specific modification and mutagenesis studies of important amino acid residues were started, resulting in the identification of an essential histidine residue (Tomme *et al.*, 1991). The present study describes the specific modification and identification of functionally important carboxy group(s).

EXPERIMENTAL

Reagents and substrates

N-Ethyl-5-phenylisoxazolium-3'-sulphonate (WRK) was purchased from Sigma (St. Louis, MO, U.S.A.). Tetranitromethane and 5,5'-dithiobis-(2-nitrobenzoic acid) were from Serva (Heidelberg, Germany). Bio-Gel P-6 was obtained from Bio-Rad (Richmond, CA, U.S.A.). 2'-Chloro-4'-nitrophenyl β -cellobioside (CNPC) was synthesized by conventional methods (De

Boeck *et al.*, 1983; Claeysens, 1988). Subtilisin was from Boehringer (Mannheim, Germany). All other substrates, ligands or chemicals (analytical grade) were prepared in our laboratory or obtained commercially.

Enzyme purification

EGD was isolated as described by Joliff *et al.* (1986a). Further purification was obtained by an affinity-chromatographic step (van Tilbeurgh *et al.*, 1984; Tomme *et al.*, 1988). All operations were performed at 4 °C in 50 mM- Na_2HPO_4 /citric acid buffer, pH 6.4 (PC-buffer). Soluble EGD was obtained after sonication and centrifugation of an overnight *E. coli* (JM 101) culture (pCT 603 plasmid), followed by streptomycin sulphate precipitation and heat treatment (60 °C, 10 min) (Joliff *et al.*, 1986a). The enzyme (20–50 mg) was recovered in the supernatant and, after addition of 10 mM-glucono- δ -lactone to inhibit β -D-glucosidase activity, this solution was applied on an affinity column (1 cm $\times$ 10 cm) consisting of *p*-aminobenzyl 1-thio- β -cellobioside coupled to Sepharose 4B. Bound EGD was eluted with 10 mM-cellobiose, and the combined fractions (active towards CNPC) were dialysed twice against distilled water (PM-1 membranes; Amicon), concentrated (10 mg/ml) and stored at –20 °C. The homogeneity of this preparation was checked by SDS/PAGE (Laemmli *et al.*, 1970) and isoelectric focusing (van Tilbeurgh & Claeysens, 1985). The absorption coefficient at 280 nm (ϵ 126000 $\text{M}^{-1} \cdot \text{cm}^{-1}$) of EGD was calculated (Beaven & Holliday, 1959) from the tryptophan and tyrosine contents (Joliff *et al.*, 1986a).

Activity measurements

EGD activity was measured at 25 °C in 50 mM-PC-buffer with 2 mM-CNPC as substrate (Claeysens, 1988). The release of 2-chloro-4-nitrophenol (pK 5.5) was monitored continuously (Perkin–Elmer B55 spectrophotometer) at 405 nm (ϵ 16000 $\text{M}^{-1} \cdot \text{cm}^{-1}$ at pH 6.4).

Abbreviations used: CNPC, 2'-chloro-4'-nitrophenyl β -cellobioside; EGD, *Clostridium thermocellum* endoglucanase D; PC-buffer, Na_2HPO_4 /citric acid buffer, pH 6.4; WRK, *N*-ethyl-5-phenylisoxazolium-3'-sulphonate; Avicel PH 101, microcrystalline cellulose (FMC Corp., Philadelphia, PA, U.S.A.).

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Enzyme modification

An aliquot of a freshly prepared stock of WRK (250 mM in 1 mM-HCl) (Petra, 1971) was diluted 1:2 in 50 mM-Mes/NaOH buffer, pH 5.5, and added to an EGD solution (0.5–1.0 μ M). Final WRK concentrations were 0.4–3.0 mM. The pH of the stirred reaction mixture (20 °C) was kept constant (0.1 M-NaOH). At indicated times, 20 μ l aliquots were diluted in 400 μ l of PC-buffer and the residual activities determined by adding 400 μ l of CNPC (4 mM) as described above.

Inactivation of EGD supplemented with ligands before (5–10 min) the addition of WRK was performed similarly.

Modifications with other reagents were performed as follows: histidine residues with diethyl pyrocarbonate (Miles, 1977); tyrosine with tetranitromethane (Riordan & Vallee, 1972) and cysteine with 5,5'-dithiobis-(2-nitrobenzoic acid) (Habeeb, 1972).

Isolation of WRK-modified enzyme and peptide analysis

EGD samples (20 μ M) in 50 mM-Mes/NaOH, pH 5.5, were treated (20 °C) with WRK (50 mM) in the absence or presence of cellobiose (0.2 M). After quenching with sodium acetate (250 mM final concn.) the mixture was applied immediately on a Bio-Gel P-6 column (1 cm $\times$ 20 cm) equilibrated and eluted (1 ml $\cdot$ min⁻¹) with either 50 mM-PC-buffer or 10 mM-Tris/HCl buffer, pH 8.0. U.v. difference spectra of the protein fractions (1 ml) were recorded (Uvikon 810; Kontron, Rotkreuz, Switzerland), and the degree of modification was determined from the 340/280 nm absorbancy ratios using an ϵ_{340} value of 7000 M⁻¹ $\cdot$ cm⁻¹ at pH 8.0 (Sinha & Brewer, 1986).

Modified enzyme (in 500 μ l of Tris/HCl, pH 8.0 buffer) was treated subsequently with 0.2 g of guanidinium chloride and 4 mg of dithiothreitol (6 min, 33 °C). This mixture (300–800 μ g of protein) was incubated with 3–8 μ g of subtilisin (Boehringer) for 10–15 min at 33 °C. Samples (150 μ g hydrolysates) were immediately injected on a C4-Vydac 214 TP-54 column (0.46 cm $\times$ 25 cm) and eluted at 1 ml $\cdot$ min⁻¹ (Waters Chromatographic System) with a linear gradient of 0.05 % trifluoroacetic acid (solvent A) and 70 % acetonitrile/0.05 % trifluoroacetic acid (solvent B). Eluents were monitored at 214 nm and 340 nm (Waters 440 detector). Selected peak fractions (340 nm) were collected and freeze-dried. Amino acid analysis was performed on a 420A Derivatizer with on-line detection of the phenylthiocarbonyl derivatives on a 130A Separation System (Applied Biosystems, Foster City, CA, U.S.A.). N-Terminal sequencing was carried out on a 477A Pulsed Liquid Sequencer (Applied Biosystems) (Benson & Hare, 1975; Hewick *et al.*, 1981).

RESULTS AND DISCUSSION

Kinetics of inactivation

At 20 °C EGD is rapidly inactivated (> 95 %) by excess WRK (800–6000-fold) in 50 mM-Mes/NaOH buffer, pH 5.5. Inactivation follows pseudo-first-order kinetics (Fig. 1a). A plot of the inactivation constants versus modifier concentration indicates saturation kinetics (Fig. 1b), and the reaction probably proceeds with prior formation of a reversible enzyme-WRK complex (Petra, 1971). Linearization (Fig. 1c) yields (i) the apparent reagent-binding constant ($K = 244$ M⁻¹), (ii) the first-order rate constant at saturation of modifier ($k_{\max} = 0.89$ min⁻¹) and (iii) the second-order rate constant ($k = 216$ M⁻¹ $\cdot$ min⁻¹).

Effects of pH and ligands

The pH-dependence of inactivation (Fig. 2) points towards the presence of an essential (carboxy) group with a pK_a of about 5.8. Similar results were obtained when EGD was inactivated with carbodi-imides (P. Tomme, unpublished work). In comparison,

hydrolysis rates for soluble substrates (CNPC) and carboxymethylcellulose are optimal at respectively pH 5.0 (Fig. 2) and pH 6.0 (Joliff *et al.*, 1986a).

Cellobiose ($K_i = 1.77$ mM) and cellotriose ($K_m = 1.47$ mM), respectively competitive inhibitor and co-substrate for CNPC hydrolysis, protect effectively against inactivation (85 and 79 % respectively at 10 mM).

Fig. 1. Inactivation of EGD by WRK

(a) Inactivation as a function of time and inactivator concentration. EGD (0.5 μ M) was incubated with various concentrations of WRK [0.42 mM ($\square$), 0.75 mM ($\blacklozenge$), 1.33 mM ($\bullet$), 2.08 mM ($\diamond$) and 3.00 mM ($\blacksquare$)] in 50 mM-Mes/NaOH buffer, pH 5.5 (20 °C). Samples were withdrawn at indicated time intervals for measurement of residual activity as described under the Experimental section. (b) Effect of the reagent concentration on the inactivation rate constant. The pseudo-first-order constants (k_{app}) were obtained from the data in Fig. 1(a). (c) Linearized plot.

Fig. 2. pH-dependence of CNPC hydrolysis rates and of inactivation rates

○, Initial velocities (v) for EGD ($0.6 \mu\text{M}$)-catalysed hydrolysis (25°C) of CNPC (2 mM) in sodium phosphate/citric acid (McIlvain's) buffer, pH 2.0–8.0, were determined as described in the Experimental section. ■, EGD ($0.5 \mu\text{M}$) was inactivated (20°C) with WRK (0.75 mM) in 50 mM -Mes buffer adjusted to the indicated pH. k_{app} values were obtained as in Fig. 1.

Fig. 3. Spectra of native (1) and WRK modified EGD in the presence (2) or absence (3) of cellobiose

EGD was modified and subsequently treated as described in the Experimental section.

Stoichiometry and specificity of the modification reaction

The characteristic maximum at 340 nm (pH 8.0) in the u.v. spectra of modified EGD (Fig. 3) enables the quantification of modified carboxy groups provided that thiol groups are not reacting (Petra, 1971; Sinha & Brewer, 1986). Evidence for the presence of three cysteine residues and a single disulphide bond were obtained by modification with 5,5'-dithiobis-(2-nitrobenzoic acid) under denaturing conditions (3 M -guanidinium chloride). EGD could not, however, be modified with this reagent under 'native' conditions (M. Claeysens, unpublished work).

As estimated from the $340/280 \text{ nm}$ absorbancy ratios, the

Fig. 4. Reversed-phase h.p.l.c. peptide mapping of EGD

EGD ($20 \mu\text{M}$) was treated with WRK (50 mM , pH 5.5, 20°C) in the absence (a) or presence (b) of cellobiose (0.2 M). Proteolysis (subtilisin) and h.p.l.c. analysis were performed as described in the Experimental section. Peaks I and I' were collected and used for subsequent analysis (see the Experimental section).

number of carboxy groups modified in the absence or presence of cellobiose (residual activity 17 or 89%), was respectively 8.1 and 7.3 (Fig. 3). A competitive inhibitor thus clearly protects one residue against modification with WRK.

The protection by cellobiose against inactivation and modification with WRK, the absence of sulphhydryl group modification and the kinetics of inactivation (saturation with respect to WRK) are cumulative evidence for the involvement of an active-site carboxy group in EGD.

The specificity of the reaction described above was further corroborated by the results of modifications with other amino-acid-specific reagents. A 5- or 10-fold excess of tetranitromethane over the total amount of tyrosine residues causes no loss of activity (P. Tomme, unpublished work), whereas EGD is partially inactivated (30% residual activity) with diethyl pyrocarbonate (Tomme *et al.*, 1991).

Isolation of an active-site peptide

After proteolysis (subtilisin) and reversed-phase chromatography with specific detection at 340 nm (Sinha & Brewer, 1986;

Fig. 5. Sequence alignment of the C-terminal regions of EGD and endo-1,4-β-glucanases from family E (Henrissat *et al.*, 1989)

Clo.th. EGD, *Clostridium thermocellum* endoglucanase D (Joliff *et al.*, 1986a); *Ps.flu.* EGA, *Pseudomonas fluorescens* var. *cellulosa* endoglucanase A (Hall & Gilbert, 1988); *Cel.fi.* CenC, *Cellulomonas fimi* endoglucanase C (Coutinho *et al.* 1991); *Bu.fi.* Ced1, *Butyrivibrio fibrisolvens* H17c celloextrinase (Berger *et al.* 1990); *P.amer.* EG, *Persea americana* (avocado) endoglucanase (Tucker *et al.* 1987); *Ph.vul.* EG, *Phaseolus vulgaris* (bean) abscission endoglucanase (Tucker & Milligan, 1991); *Di.dis.* SGSP, *Dictyostelium discoideum* spore-germination-specific protein (Giorda *et al.*, 1990); *Th.fus.* E4, *Thermomonospora fusca* endoglucanase 4 (Lao *et al.*, 1991); *Cel.fi.* CenB, *Cellulomonas fimi* endoglucanase B (Meinke *et al.*, 1991) *Clo.st.* EGZ, *Clostridium stercorarium* endoglucanase Z (Jauris *et al.*, 1990). The sequences are numbered from the start of the signal sequences. :, identical amino acids; *, conservative changes; —, gaps introduced to improve the alignment. The sequence of peptide I (I') (Fig. 4) corresponds to aa 508–518 (—). The region tentatively deduced from the amino acid composition is also indicated (—).

Tomme & Claeysens, 1989), a peptide containing modified residue(s) could be isolated (Fig. 4). Peptide maps (214 nm tracings) of enzyme samples, modified in the absence or presence of a protector (cellobiose) coincide. The presence of a peak (I) in the 340 nm tracing and its absence in the protected sample (I') (Fig. 4) provide proof for the implication of (an) active site residue(s). The results of N-terminal sequencing analysis (ten cycles) were identical for the corresponding peptides (I and I'), and the deduced sequence (508–518) was located in the EGD primary structure (Joliff *et al.*, 1986a) (Fig. 5). Amino acid analysis (Table 1) produces tentative evidence for the presence of

a rather long peptide, its sequence probably extending to residue 562. It contains several carboxy groups (five Asp and two Glu) which could potentially have reacted with WRK.

Alignment with other cellulase sequences

Since further analysis of the isolated peptide was hampered by the instability of the label (Sinha & Brewer, 1986), an extensive comparison of sequences in cellulases belonging to Family E (Henrissat *et al.*, 1989) was started (Table 2). This family can be divided further into two subgroups, as was proposed by Béguin (1990). Endoglucanases from both bacteria and plants are

Table 1. Amino acid composition of peptide I (I')

Comparison of the experimental determined amino acid composition for peptide I (I') (Fig. 4) with that expected for the putative active-centre peptide 508–562 (Fig. 5).

Amino acid	Number of amino-acids in peptide (508–562)	
	Found	Expected
Asparagine	9.3	10
Glutamine	4.2	4
Serine	1.0	2
Glycine	7.9	8
Histidine	1.0	1
Arginine	1.0	2
Threonine	2.1	1
Alanine	2.8	3
Proline	7.5	7
Tyrosine	1.8	2
Valine	1.5	2
Methionine	0.5	1
Cysteine	0.0	0
Isoleucine	2.6	5
Leucine	1.1	1
Phenylalanine	2.1	0
Lysine	1.1	1

Table 2. Composition of cellulase family E

The Table shows a classification of the current members of family E (Henrissat *et al.*, 1989) into subgroups E1 and E2 as proposed by Béguin (1990). Abbreviation: SGSP, spore-germination-specific protein.

Enzyme	Organism	Reference
Subgroup E1		
Ced1	<i>Butyrivibrio fibrisolvens</i>	Berger <i>et al.</i> (1990)
CenC	<i>Cellulomonas fimi</i>	Coutinho <i>et al.</i> (1991)
EGD	<i>Clostridium thermocellum</i>	Joliff <i>et al.</i> (1986)
EG	<i>Pseudomonas fluorescens</i>	Hall & Gilbert (1988)
Subgroups E2		
CenB	<i>Cellulomonas fimi</i>	Meinke <i>et al.</i> (1991)
EGF	<i>Clostridium thermocellum</i>	Béguin (1990)
EGZ	<i>Clostridium stercorarium</i>	Jauris <i>et al.</i> (1990)
SGSP	<i>Dictyostelium discoideum</i>	Giorda <i>et al.</i> (1990)
EndA	<i>Fibrobacter succinogenes</i>	Cavicchioli <i>et al.</i> (1991)
EG	<i>Persea americana</i>	Tucker <i>et al.</i> (1987)
Cell1	<i>Persea americana</i>	Cass <i>et al.</i> (1990)
Cell2	<i>Persea americana</i>	Cass <i>et al.</i> (1990)
EG	<i>Phaseolus vulgaris</i>	Tucker & Milligan (1991)
E4	<i>Thermomonospora fusca</i>	Lao <i>et al.</i> (1991)

present. Only a small number of regions are conserved between all members and, from the alignment of the isolated EGD peptide with the corresponding sequences of nine other endoglucanases, it is apparent that Asp-546 and Glu-555 are conserved (Fig. 5), suggesting strongly their (functional/catalytic) importance. The sequence identity in this region is visibly higher than for the rest of the protein. EGD from *Clostridium thermocellum* shows the highest degree of overall identity (about 27%) with EGA from *Pseudomonas fluorescens* (Henrissat *et al.*, 1989), whereas in the corresponding 508–562 (EGD numbering) region it amounts to 57%. Similarly the overall identity between primary structures of all the enzymes in family E (Fig. 5) is calculated to be approx. 6%, whereas in the putative active-centre region this is three times higher (19%). This is supporting evidence for the

importance of the 508–562 region, and recently a point-mutation study has led to the identification of Glu-555 as the most likely catalytic residue (Chauvaux *et al.* 1992). The presence of an essential histidine residue (His-516) in the same region was shown in a previous study (Tomme *et al.*, 1991). It highlights the functional and/or structural importance of the C-terminal region of EGD.

Cellulases Cell1 and Cell2 from *Persea americana* (avocado), being very similar to endoglucanase (EG) from the same organism were not aligned. EGF from *Clostridium thermocellum* and endoglucanase EndA from *Fibrobacter succinogenes* are also left out. The sequence of the former has not yet been published, but was classified by Béguin (1990) in subgroup E2. EndA, although containing the His-516 (EGD numbering), is the only endoglucanase from this family apparently lacking the corresponding 520 → C-terminus region. The cloned *endA*-gene fragment, however, encodes two overlapping open reading frames and a frameshift had to be introduced to allow a contiguous synthesis of the endoglucanase (Cavicchioli *et al.*, 1991). In view of our results, a careful re-examination of the *endA* gene and biochemical characterization of its product might be worthwhile.

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REFERENCES

- Beaven, G. H. & Holliday, E. R. (1959) *Adv. Protein Chem.* **7**, 319–386
- Béguin, P. (1990) *Annu. Rev. Microbiol.* **44**, 219–248
- Benson, J. R. & Hare, P. E. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 619–622
- Berger, E., Jones, W. A., Jones, D. T. & Woods, D. R. (1990) *Mol. Gen. Genet.* **223**, 310–318
- Cass, L. G., Kirven, K. A. & Christoffersen, R. E. (1990) *Mol. Gen. Genet.* **223**, 76–86
- Cavicchioli, R., East, P. D. & Watson, K. (1991) *J. Bacteriol.* **173**, 3265–3268
- Chavaux, S., Béguin, P. & Aubert, J. P. (1992) *J. Biol. Chem.* **267**, 4472–4478
- Claeyssens, M. (1988) *FEMS Symp.* **43**, 393–397
- Coutinho, J. B., Moser, B., Kilburn, D. G., Warren, R. A. J. & Miller, R. C. Jr. (1991) *Mol. Microbiol.* **5**, 1221–1233
- De Boeck, H., Matta, K. L., Claeyssens, M., Sharon, N. & Loontjens, F. (1983) *Eur. J. Biochem.* **131**, 453–460
- Giorda, R., Ohmachi, T., Shaw, D. R. & Ennis, H. L. (1990) *Biochemistry* **29**, 7264–7269
- Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 457–463
- Hall, J. & Gilbert, J. G. (1988) *Mol. Gen. Genet.* **213**, 112–117
- Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L. & Mornon, J. P. (1989) *Gene* **81**, 83–95
- Hewick, R. M., Hunkapiller, M. W., Hool, L. E. & Dreyer, W. J. (1981) *J. Biol. Chem.* **256**, 7990–7997
- Jauris, S., Rücknagel, K. P., Schwarz, W. H., Kratzsch, P., Bronnenmeier, K. & Staudenbauer, W. L. (1990) *Mol. Gen. Genet.* **223**, 258–267
- Joliff, G., Béguin, P. & Aubert, J. P. (1986a) *Nucleic Acids Res.* **14**, 8605–8613
- Joliff, G., Béguin, P., Juy, M., Millet, J., Ryter, A., Poljak, R. & Aubert, J. P. (1986b) *Bio/Technology* **4**, 896–900
- Joliff, G., Béguin, P., Millet, J., Aubert, J. P., Alzari, P., Juy, M. & Poljak, R. J. (1986c) *J. Mol. Biol.* **189**, 249–250
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lao, G., Ghangas, G. S., Jung, E. D. & Wilson, D. B. (1991) *J. Bacteriol.* **173**, 3397–3407
- Meinke, A., Braun, C., Gilkes, N. R., Kilburn, D. G., Miller, R. C. & Warrne, R. A. J. (1991) *J. Bacteriol.* **173**, 308–314
- Miles, E. W. (1977) *Methods Enzymol.* **47**, 431–442

- Mitsuishi, Y., Nitrisinpraset, S., Saloheimo, M., Biese, I., Reinikainen, T., Claeysens, M., Keränen, S., Knowles, J. K. C. & Teeri, T. (1990) *FEBS Lett.* **275**, 135–138
- Petra, P. H. (1971) *Biochemistry* **10**, 3163–3170
- Py, B., Bortoli-German, I., Haiech, J., Chippaux, M. & Barras, F. (1991) *Protein Eng.* **4**, 325–333
- Riordan, J. F. & Vallee, B. L. (1972) *Methods Enzymol.* **25**, 515–521
- Sinha, U. & Brewer, J. M. (1972) *Anal. Biochem.* **151**, 327–333
- Sinha, U. & Brewer, J. M. (1986) *Biochem. Cell Biol.* **64**, 970–975
- Tomme, P. & Claeysens, M. (1989) *FEBS Lett.* **243**, 239–243
- Tomme, P., McCrae, S., Wood, T. M. & Claeysens, M. (1988) *Methods Enzymol.* **160**, 187–193
- Tomme, P., Chauvaux, S., Béguin, P., Millet, J., Aubert, J. P. & Claeysens, M. (1991) *J. Biol. Chem.* **266**, 10313–10318
- Tucker, M. L. & Milligan, S. B. (1991) *Plant Physiol.* **195**, 928–933
- Tucker, M. L., Durbin, M. L., Clegg, M. T. & Lewis, L. N. (1987) *Plant Mol. Biol.* **9**, 197–203
- van Tilbeurgh, H. & Claeysens, M. (1985) *FEBS Lett.* **187**, 283–288
- van Tilbeurgh, H., Pettersson, L. G., Bhikhabhai, R. & Claeysens, M. (1984) *FEBS Lett.* **187**, 283–288

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