# Purification and characterisation of epithiospecifier protein from *Brassica napus*: enzymic intramolecular sulphur addition within alkenyl thiohydroximates derived from alkenyl glucosinolate hydrolysis

Hooi L. Foo[a], Line M. Grønning[a,b], Lucy Goodenough[a], Atle M. Bones[b], Brit-Eli Danielsen[a,b], Don A. Whiting[c], John T. Rossiter[a,*]

[a] *Department of Biological Sciences, Wye College, University of London, Wye, Ashford, Kent TN25 5AH, UK*
[b] *Unigen Center for Molecular Biology, Norwegian University for Science and Technology, MTFS, N-7005 Trondheim, Norway*
[c] *Department of Chemistry, University of Nottingham, Nottingham NG7 2RD, UK*

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**Abstract Epithiospecifier protein (ESP), a ferrous ion dependent protein, has a potential role in regulating the release of elemental sulphur, nitriles, isothiocyanates and cyanoepithioalkanes from glucosinolates. Two classes of ESP polypeptides were purified with molecular masses of 39 and 35 kDa, and we show that the previously reported instability was conditionally dependent. The 39 kDa polypeptide was made up of two distinct isozymes (5.00, 5.14) whilst several were present for the 35 kDa form of ESP (5.40–5.66). An anti-ESP antibody reacted with both the 39 and 35 kDa ESP forms in *Brassica napus* and strongly with a polypeptide corresponding to the 35 kDa ESP form in *Crambe abyssinica*, but did not detect any ESP in *Sinapis alba* or *Raphanus sativus*. A cytochrome P-450 mediated iron dependent epoxidation type mechanism is suggested for ESP.**



*Key words:* Myrosinase; Epithiospecifier protein; Thioglucoside (glucosinolate); Thioglucoside glucohydrolase (EC 3.2.3.1 myrosinase); *Brassica napus*

## 1. Introduction

Glucosinolates (thioglucosides) are a class of sulphur containing secondary metabolites present in all cruciferous plants [1]. Glucosinolate hydrolysis is catalysed by myrosinases (thioglucoside glucohydrolases) to give a range of products such as isothiocyanates (or oxazolidine-2-thiones if a β hydroxy group is present), nitriles, cyanoepithioalkanes, sulphate, sulphur and glucose, some of which have important roles in the plant's defence against pests and diseases [2,3]. Glucosinolates and their degradation products are important bioactive compounds in the diet and health of both animals and humans [2].

Myrosinase catalyses hydrolysis of glucosinolates to give rise to an unstable thiohydroximate-*O*-sulphonate which can be rearranged to an isothiocyanate. Alternatively, if epithiospecifier protein (ESP) and ferrous ions are present then cyanoepithioalkanes are produced. ESP appears to regulate the nature of the degradation products derived from the hydrolysis of alkenyl glucosinolates by the enzyme myrosinase. ESP is unique inasmuch as it has no activity towards the initial substrate but only against the unstable thiohydroximate-*O*-sulphonate intermediate (Fig. 1).

The organisation and biochemistry of the myrosinase enzyme system has been reviewed by Bones and Rossiter [2]. The X-ray crystallographic structure of a plant myrosinase from *Sinapis alba* has recently been published [4] together with mechanistic studies. However, very little is known about the characteristics of ESP.

The oilseed rape plant is ideal for biotechnological development. It is an important plant for oil and as a source of protein for use in livestock production. More recently, the potential for recovering glucosinolates as a raw material for the generation of biocides and as a starting material for chemical synthesis has been explored [5]. 2-Hydroxybut-3-enylglucosinolate is the major glucosinolate in seeds of *Brassica napus* and *Crambe abyssinica*. It is present as (2-*R*)-2-hydroxybut-3-enylglucosinolate (progoitrin) in *B. napus* and as (2-*S*)-2-hydroxybut-3-enylglucosinolate (epiprogoitrin) in *C. abyssinica*. *C. abyssinica* is at present being considered as a new source of plant oils for the EU market. On hydrolysis of progoitrin in the presence of $Fe^{2+}$/ESP, the corresponding 4,5-epithio-3-hydroxypentanenitriles (1, 2) and 1-cyano-2-hydroxybut-3-ene (3) are formed while in the absence of $Fe^{2+}$/ESP, the corresponding 5-vinyloxazolidine-2-thione (goitrin) is formed (4) (Fig. 2). Similarly, epiprogoitrin, the opposite isomer of progoitrin, will give rise to stereoisomers of the opposite configuration.

ESP [6] from *C. abyssinica* has previously been described as an unstable protein (with a short half-life) that requires sonication and is dependent on ferrous ions for activity. Since ESP is of biological importance we have set out to purify and characterise this potentially important protein.

## 2. Materials and methods

### 2.1. ESP assay

Assays were carried out by incubating ESP fractions in an assay mixture containing ferrous ions (0.6 mM), imidazole–HCl buffer (50 mM), progoitrin (5.2 mM) and myrosinase (0.47 mg from a partially purified preparation from *Sinapis alba* seed). ESP activity was determined by measuring the 4,5-epithio-3-hydroxypentanenitriles and 1-cyano-2-hydroxy-3-butene by capillary GC (12 m PEG column, Hewlett-Packard 5880A). Products were confirmed by GC-MS (JEOL JMS/AX505W).

### 2.2. Purification of ESP

*Brassica napus* var. *napobrassica* cv. Laurentian seed (80 g) was

*Corresponding author. Fax: (44)-1233-813140.
E-mail: j.rossiter@wye.ac.uk


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defatted, glucosinolates removed and the protein extracted into 500 ml 0.1 M imidazole–HCl buffer (pH 6.8, 1 mM DTT, 1 mM PMSF, 10% w/v PVP). Following centrifugation, a 40–90% saturated ammonium sulphate fraction was prepared and the dissolved precipitate dialysed against 20 mM imidazole–HCl buffer (pH 6.8). The protein was then passed through an S-Sepharose column equilibrated with 20 mM imidazole–HCl buffer (pH 6.8). Bound protein (without ESP) was eluted from the S-Sepharose with 0.2 M sodium chloride. The unbound protein containing ESP activity was further purified by gel filtration on an S-100 Sephacryl column (2.6×60.0 cm). Active fractions from the gel filtration column were analysed by SDS–PAGE electrophoresis and further purified by anion exchange chromatography on a MonoQ HR 5/5 column. Repeated ion exchange chromatography gave two active proteins with molecular masses of 35 and 39 kDa.

### 2.3. Gel electrophoresis

For SDS–PAGE separation, seed protein was extracted in imidazole–HCl buffer (0.1 M, pH 6.8) containing 1 mM DTT, 1 mM PMSF, EDTA-free protease inhibitor cocktail (Boehringer Mannheim) and 10% w/v PVP). Ammonium sulphate fractions were made (40–90% saturation) and the pellets reconstituted in 0.1 M imidazole–HCl buffer (pH 6.8) containing 1 mM DDT and EDTA-free protease inhibitor cocktail (Boehringer Mannheim) and desalted on a Bio-Rad Econo-Pac 10 DG desalting column against 20 mM imidazole–HCl buffer (pH 6.8) containing the protease inhibitor cocktail. Polypeptides were resolved in 13.5% (w/v) acrylamide vertical slab gels according to the procedure of Laemmli [7]. Polypeptides were stained with Coomassie blue R-250 or analysed for carbohydrate content with the periodic acid-Schiff stain [8].

Isoelectric focusing was performed on a commercially available Ampholine™ PAGplate, pH 3.5–9.5 (Amersham Pharmacia Biotech AB) according to the manufacturer's instructions.

### 2.4. Preparation of antibodies

Polyclonal antibodies were raised to the 39 kDa form of ESP in New Zealand White rabbit by subcutaneous injections of 50 μg of pure protein in Freund's complete adjuvant followed by booster injections in Freund's adjuvant. For immunoblots, crude plant protein and purified polypeptide preparations were Western blotted onto nitrocellulose filter membrane after SDS–PAGE [9]. Polypeptides were transferred in 2 h using a press blot [10]. The primary antibody was used at a 1:2000 dilution. Protein–antibody complexes were detected with alkaline phosphatase linked goat anti-rabbit IgG, at a dilution of 1:10 000.

## 3. Results and discussion

Previous work on the purification of ESP by Tookey [6] was hindered by the perceived instability of the protein together with use of ferrous ions and acetate buffer throughout the purification procedure. Using the same Tookey [6] protocol we also found ESP to be apparently unstable. However, further work showed that the Tookey [6] protocol caused ESP to be precipitated in a complex with ferric ions at pH 5.8 (near to the p*I* of ESP) thus explaining its perceived instability. We found that using imidazole–HCl buffer at pH 6.8 resulted in the retention of activity throughout the purification process provided ferrous ions were omitted. Ferrous ions were only necessary in the assay.

Table 1
Purification of ESP from seeds of *Brassica napus* var. *napobrassica* cv. Laurentian

| | Total protein (mg) | % CHB | % ETAs |
|---|---|---|---|
| 40–90% AS fraction | 962 | 10 | 90 |
| S-Sepharose-bound | 264 | 100 | 0 |
| S-Sepharose-unbound | 168 | 15 | 85 |
| S-100 Sephacryl fraction 19 | 5.6 | 28 | 72 |
| S-100 Sephacryl fraction 20 | 1.5 | 8 | 92 |
| MonoQ, 39 kDa (1.8 μg pure protein used in assay) | 0.200 | 86 | 14 |
| MonoQ, 35 kDa (10.0 μg pure protein used in assay) | 0.144 | 76 | 24 |

CHB = 1-cyano-2-hydroxybut-3-ene, ETAs = 4,5-epithio-3-hydroxypentanenitriles, AS = ammonium sulphate. Progoitrin was used as substrate and activity is represented as the % of the total peak areas (determined by GC) of the ETAs and CHB.

ESP was purified in a relatively straightforward procedure to apparent homogeneity by traditional protein purification techniques (Table 1). Results are reported as the proportion of hydroxy-cyanoepithioalkanes to the corresponding nitrile because there is currently no suitable assay for ESP activity other than by direct analysis of the products. On the basis of gel filtration the molecular mass of ESP has been shown to be 30–40 kDa [6]. Partially pure ESP (S-Sepharose ion exchange chromatography followed by gel filtration on Sephacryl S-100) was further purified into two major fractions by repeated chromatography (Fig. 3), to give single homogeneous peaks (data not shown) on a MonoQ ion exchange column. SDS–PAGE analysis (Fig. 3) of each fraction showed ESP to comsist of 35 and 39 kDa (±2 kDa) polypeptides. Isoelectric focusing analysis of each fraction shows the ESP with a mass of 35 kDa to be comprised of a number of isoforms (Fig. 4) with p*I*s of 5.40–5.66 and a possible minor impurity

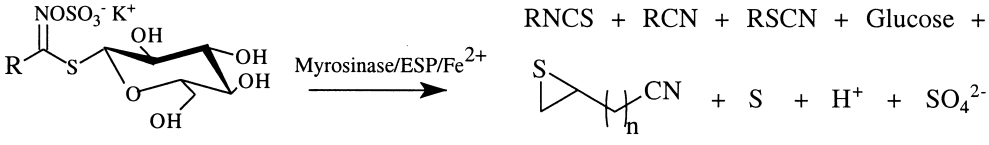


Fig. 1. Hydrolysis products of glucosinolates.

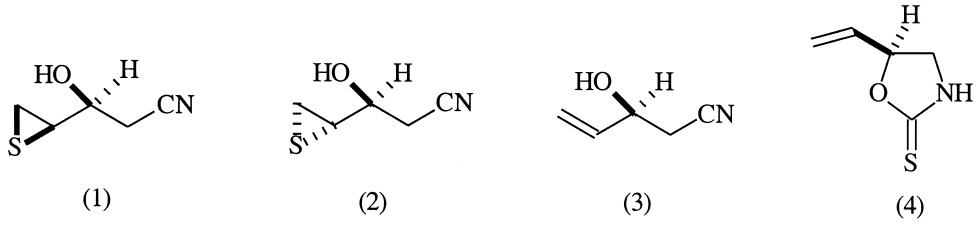


Fig. 2. Hydrolysis products of the glucosinolate progoitrin.

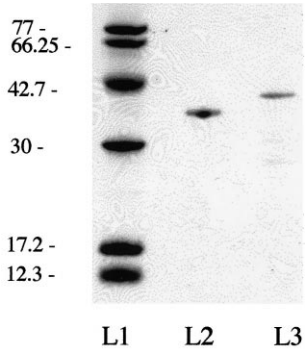


Fig. 3. SDS–PAGE of purified ESP fractions. L1, BDH $M_r$ markers, ovotransferrin (77 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (30 kDa), myoglobin (17.2 kDa) and cytochrome *c* (12.3 kDa); L2, ESP, 35 kDa ±2 kDa; L3, ESP, 39 kDa ±2 kDa.

with a p*I* of 5.25. The ESP with a mass of 39 kDa was made up of two (Fig. 4) distinct isoforms with p*I*s of 5.00 and 5.14. The reduction in ESP activity at the final purification step may be explained by possible protein dilution effects and further work is required to optimise storage conditions.

Taipalensuu et al. [11] have suggested that ESP is the myrosinase associated protein (MyAP). MyAP is a glycoprotein, purified from *Brassica napus*, associated with myrosinase complexes precipitated with anti-myrosinase antibodies. It is wound and methyl jasmonate inducible and has similarities to an early nodulin. However, no activity has been demonstrated for this protein. Unlike myrosinase, ESP does not bind to concanavalin A and unlike MyAP it does not bind to lentil lectin. In addition, analysis (Fig. 5) of the carbohydrate content of ESP forms with the periodic acid-Schiff reagent showed no staining in comparison to the 77 kDa myrosinase which is heavily glycosylated. Thus, the current evidence seems to indicate that ESP is not MyAP. We have also shown that ESP does not require any specific myrosinase (data not shown) as ESP is also active with myrosinase isolated from the aphid *Brevicoryne brassicacae*.

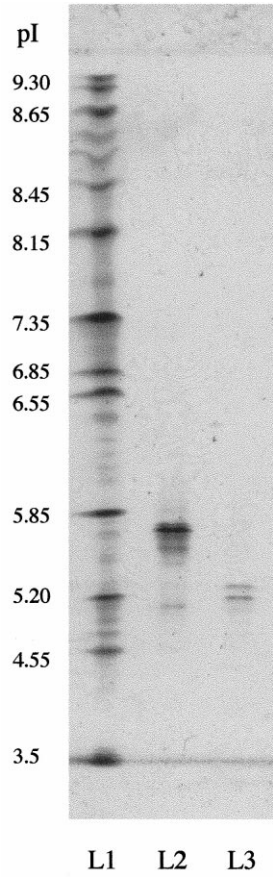


Fig. 4. Isoelectric focusing of purified ESPs. 3 μg of each purified fraction was applied to the gel. Proteins were stained with Coomassie blue R-250. L3, 39 kDa ESP; L2, 35 kDa ESP; L1, p*I* marker proteins (Pharmacia), trypsinogen (9.30), lentil lectin-basic (8.65), lentil lectin-middle (8.45), lentil lectin-acidic (8.15), myoglobin-basic (7.35), myoglobin-acidic (6.85), human carbonic anhydrase (6.55), bovine carbonic anhydrase (5.85), β-lactoglobulin A (5.20), soybean trypsin inhibitor (4.55), amyloglucosidase (3.50).


Fig. 5. Gel stained for carbohydrate with periodic acid-Schiff reagent. L1, 3 μg pure myrosinase from *Sinapis alba* (77 kDa form); L2, 3 μg ESP, 35 kDa ±2 kDa; L3, 3 μg ESP, 39 kDa ±2 kDa.

A polyclonal antibody was raised to the 39 kDa ESP polypeptide. This antibody to the 39 kDa ESP also cross-reacted with the 35 kDa form of ESP (Fig. 6). Although the affinity to 35 kDa ESP seems to be lower it appears that they must have some similar epitopes. Some cruciferous plants are known to lack ESP activity while some [6] such as *C. abyssinica* are rich in activity. Western blot analysis with the anti-ESP antibody was carried out on protein extracts of four different crucifers.

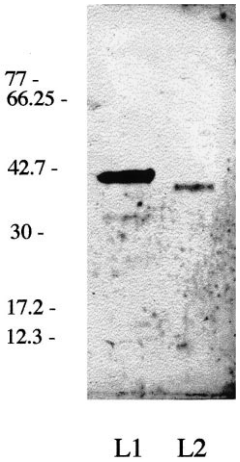


Fig. 6. Western blot analysis of purified ESP fractions with anti-ESP antibodies. L1, 39 kDa ESP; L2, 35 kDa ESP.

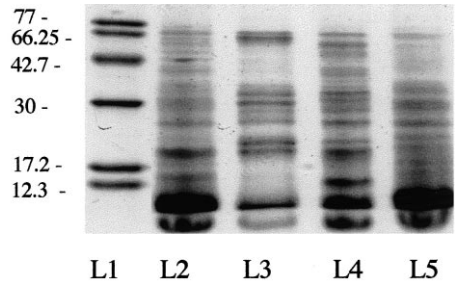


Fig. 7. SDS–PAGE of 40–90% ammonium sulphate fractions (20 μg/lane) from L2, *Brassica napus* var. *napobrassica* cv. Laurentian; L3, *Sinapis alba*; L4, *Raphanus sativus*; L5, *Crambe abyssinica*; L1, BDH $M_r$ markers, ovotransferrin (77 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (30 kDa), myoglobin (17.2 kDa) and cytochrome *c* (12.3 kDa).

The SDS–PAGE analysis is shown in Fig. 7 and the corresponding Western blots in Fig. 8. The anti-ESP antibody reacted with both the 39 and 35 kDa ESP forms in *B. napus* var. *napobrassica* cv. Laurentian and strongly with a polypeptide corresponding to the 35 kDa ESP form in *C. abyssinica*. *S. alba* and *Raphanus sativus* seeds, which have previously been reported to lack ESP activity [12], showed no cross-reaction with the anti-ESP antibody. Thus it would appear that *B. napus* var. *napobrassica* cv. Laurentian contains two main polypeptide forms while *C. abyssinica* contains just one. Protease inhibitors were added to the extraction buffers ruling out the possibility of the 35 kDa ESP polypeptide being a proteolytic degradation product of the 39 kDa ESP form. It is apparent from the purification data that ESP is not an abundant protein. However, the anti-ESP antibodies stain the ESP in crude extracts of the seed material although some background is present.

Previous work by Brocker and Benn [13] has shown that the sulphur of the cyanoepithioalkanes is derived from the β-thioglucosidic link of alkenyl glucosinolates. Mechanistically, ESP is possibly analogous to a cytochrome P-450 such as in iron dependent epoxidations [14]. For oxygen transfer, $Fe^{III}/Fe^{IV}$ is necessary while sulphur transfer may be possible with $Fe^{II}/Fe^{III}$. However, in addition to the generation of a thiirane ring, the cyano functional group is also formed. The insertion

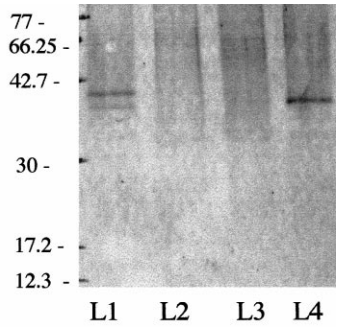


Fig. 8. Western blot analysis with anti-ESP antibodies of 40–90% ammonium sulphate fractions (20 μg/lane) from L1, *Brassica napus* var. *napobrassica* cv. Laurentian; L2, *Sinapis alba*; L3, *Raphanus sativus*; L4, *Crambe abyssinica*;

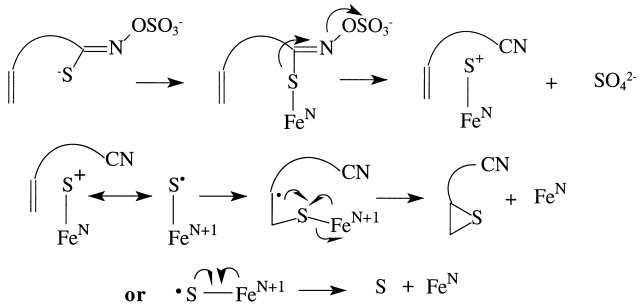


Fig. 9. Generalised mechanism for the formation of cyanoepithioalkanes and nitriles.

of sulphur is not stereospecific and a generalised mechanism for ESP is shown in Fig. 9.

## 4. Conclusion

Epithiospecifier proteins have been purified, and contrary to previous work, appear to be relatively stable proteins. Two classes of epithiospecifier protein have been purified to apparent homogeneity. ESP is likely to regulate the nature of the degradation products of the alkenyl glucosinolates. ESP, which has no known enzymatic activity of its own, can enzymatically react with a myrosinase generated unstable alkenyl thiohydroximate-*O*-sulphonate intermediate to produce a different product. This represents a unique system in eukaryotes. A mechanism for the generation of cyanoepithioalkanes with mechanistic similarity to cytochrome P-450 mediated iron dependent oxidation is suggested. ESP is likely to regulate the nature of the degradation products of the alkenyl glucosinolates and thereby the bioactive profile of important crop species.

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