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Isolation of the epithiospecifier protein from oil-rape (*Brassica napus* ssp. *oleifera*) seed and its characterization

Roberta Bernardi[a], Armando Negri[b], Severino Ronchi[b], Sandro Palmieri[a,*]

[a] *Istituto Sperimentale per le Colture Industriali, MiPAF, Via di Corticella 133, 40129 Bologna, Italy*
[b] *Istituto di Fisiologia Veterinaria, Università di Milano, Via Celoria 10, 20100 Milan, Italy*

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Abstract The epithiospecifier protein (ESP) is a myrosinase (MYR) cofactor, which is necessary to drive the MYR-catalyzed hydrolysis of some specific glucosinolates towards the production of cyanoepithioalkanes instead of isothiocyanates and nitriles. ESP was isolated from *Brassica napus* seeds by anionic exchange and gel filtration chromatography. ESP showed a molecular weight of about 39 kDa and p*I* 5.3. The amino acid sequence of several tryptic peptides of ESP (accounting for about 50% of the total sequence) made it possible to establish the high similarity (81% identity) with a hypothetical 37 kDa protein (TrEMBL data base accession number Q39104) and several jasmonate-inducible proteins from *Arabidopsis thaliana*. This observation suggests that ESP is likely to be involved in jasmonate-mediated defence and disease resistance mechanisms.

Key words: Epithiospecifier protein; Myrosinase; Cyanoepithioalkane; Rapeseed; Jasmonate

1. Introduction

Glucosinolates (GLs) are secondary plant metabolites contained mainly in Brassicaceae. These compounds are anionic thioesters containing a β-thioglucoside-type bond and a variable aliphatic, aromatic and heteroaromatic side chain. GLs can be hydrolyzed by myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) (MYR) to give a variety of bioactive degradation products such as isothiocyanates, thiocyanates, nitriles and cyanoepithioalkanes [1,2].

The MYR–GLs system is always present at different concentrations in all Brassicaceae organs where, when activated by tissue damage, it plays a defensive role against herbivores and different kinds of pathogens [3,4]. Theoretically this system can generate hundreds of aglycones which are spontaneously rearranged to give degradation products whose cytotoxicity depends on their chemical structure and reaction conditions. Among the latter, pH and the presence of Fe^{2+} and an epithiospecifier protein (ESP) are the most important [5].

ESP is a rather small labile protein which does not have any enzymatic activity per se. However, it is considered to be a necessary MYR cofactor to drive the MYR-catalyzed reaction towards the production of cyanoepithioalkanes, starting from GLs containing a double terminal bond [5]. In this regard, it is interesting to note that the plants containing ESP also contain suitable substrates (GLs with a double terminal bond) for their transformation into cyanoepithioalkanes. ESP activity was found in seeds of *Crambe abyssinica*, turnip (*Brassica campestris* ssp. *rapifera*), Brussels sprouts (*Brassica oleracea* var. *gemmifera*), white cabbage (*Brassica oleracea* var. *capitata*), etc., but not in other Brassicaceae such as *Sinapis alba* or horseradish (*Armoracia*) [6]. In addition, MacLeod and Rossiter [7], also found ESP in rapeseed (*Brassica napus* ssp. *oleifera*) and *Lepidium sativum*, although they only studied its activity in crude extracts.

The final aim of ESP characterisation was to clarify the biochemical mechanism of the GLs–MYR–ESP system and its biological role in plants. This study reports, for the first time, a purification protocol and a partial characterisation of the primary ESP structure. These data are discussed and compared with those available for other similar proteins found in the Brassicaceae.

2. Materials and methods

2.1. Plant material, treatment and extraction

Brassica napus 00 cv B013 seed meals were defatted using hexane as a solvent. Defatted meal (100 g) was homogenised with 1 l of 20 mM acetate buffer pH 5.8 containing 0.2 M NaCl, 1 mM dithiothreitol and 5% (w/w) PVP using an Ultra-Turrax homogeniser (model T25 IKA Labortechnik) in an ice bath for 15 min. The homogenate was centrifuged at 17 700×*g* for 30 min at 4°C.

2.2. MYR-ESP assay

Aliquots of fractions containing ESP and endogenous MYR were incubated with a solution containing 50 mM acetate buffer pH 5.5, 2.5 mM Fe^{2+}, 5 mM cysteine and 3 mg/ml *epi*-progoitrin (E-PRO), previously isolated from ripe *Crambe abyssinica* seeds [8], at 25°C. The total volume of the assay was 1 ml. After 30 min, the E-PRO degradation products were extracted with ethyl ether adding phenyl isothiocyanate as internal standard and analyzed with a GC-MS Hewlett-Packard Mod. G1800A GCD. The analytical conditions were as follows: capillary column, HP5-MS (30 m×0.25 mm×0.25 μm); the injector and detector temperatures were 250 and 280°C, respectively; He as the carrier gas at a flow of 1 ml/min; the temperature program as follows: starting temperature, 60°C for 4 min; final temperature, 220°C and rate, 10°C/min.

The amount of cyanoepithioalkanes produced was determined as described by Leoni et al. [9].

One ESP unit was defined as the amount of protein needed to produce 1 μmol of cyanoepithioalkanes in 1 min.

2.3. Isolation procedure

The crude extract was dialyzed against water at 4°C and centrifuged at 17 700×*g*. The supernatant was equilibrated at pH 7.5 with Tris–HCl buffer and loaded into a Q-Sepharose Fast Flow XR 26/20 column (Pharmacia), equilibrated with 25 mM Tris–HCl pH 7.5 at 4°C. The protein was eluted using a linear gradient of NaCl, in the starting buffer, from 0.0 to 0.4 M in 160 ml with a flow of 1 ml/min, using a

*Corresponding author. Fax: (39)-51-316851.

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Pharmacia FPLC System equipped with a UVICORD SII detector (Pharmacia). Fractions containing ESP were collected and dialyzed against 25 mM Tris–HCl buffer pH 7.5. After filtration the solution containing ESP was loaded into a Q-Sepharose High Performance HR 10/10 column, equilibrated with 25 mM Tris–HCl pH 7.5. The proteins were eluted using a linear gradient of NaCl, in the starting buffer, from 0.0 to 0.4 M in 100 ml with a flow of 1 ml/min. The fractions containing ESP were collected, concentrated and loaded into a FPLC-Superdex 200 HR 10/30 gel filtration column (Pharmacia), equilibrated with 25 mM Tris–HCl containing 0.15 M NaCl.

2.4. Amino acid sequence determination

Carbamidomethylation, trypsin digestion, HPLC separation of the tryptic peptides and amino acid sequences were obtained as described by Negri et al. [10].

2.5. Electrophoresis

SDS–PAGE and IEF of the isolated enzyme were performed with a Phastsystem apparatus (Pharmacia), using homogeneous Phastgel 12.5% and Phast IEF 3–9, respectively. Pharmacia standards and protocols were used.

2.6. Protein determination

The soluble protein concentration was determined following the Coomassie blue dye binding method [11].

3. Results and discussion

3.1. ESP isolation and properties

Over the last two decades, ESP presence in rapeseed and some other Brassicaceae has been postulated on the basis of its activity determined in ripe seed extracts [6,7]. ESP is a labile protein requiring a tedious and time consuming assay to determine its activity. Perhaps due to these difficulties, ESP has only been partially purified [5] but not isolated in a homogeneous form until now, although it is an intriguing protein for its crucial role in the production of the strongly cytotoxic cyanoepithioalkanes (Galletti et al., in preparation) [12]. Thus, in order to start a project to clarify its biological role, it was absolutely necessary to obtain a homogeneous preparation of the protein. The purification protocol detailed in Section 2 made it possible to obtain about 4 mg of protein starting from 100 g of defatted rapeseed meal (Table 1). The purity of the protein was assessed on the basis of the presence of a single band, both in SDS–PAGE and isoelectric focusing experiments (MW = 39 kDa, pI = 5.3). In a gel filtration experiment under native conditions, the protein shows a single

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o04317    -STIKLLGKWIKVEQKG-EGPGLRCSHGIAQVGNKIYSFGGEFTPNQPIDKHLYVFDLETRTWSISPATGDVPHLSCLGVRM 224
o04318    -STPKLLGNWIKVEQNG-EGPGLRCSHGIAQVGNKIYSFGGELIPNQPIDKHLYVFDLETRTWSIAPATGDVPHLSCLGVRM 224
o49326    -ATPKLRGKWIKVEQKG-EGPGPRCSHDIAQVGNKIFSFGGELTPNQPIDKHLYVFDLETRTWSISPATGDVPNLSCLGVRM 225
Q39104     MAPTLQGQWIKVGQKGGTGPGPRSSHGIAAVGDKLYSFGGELTPNKHIDKDLYVFDFNTQTWSIAQPKGDAPTVSCLGVRM  81
ESP                                SSHGIAVVGDKLYSFGGELTPNISIDKDLYVFDFKTHTWSIETGSGDLPNVK     M
                                    **:** **:*::*****::**  *** *****  * .****   :**:* ::     *

o04317    VSVGSTLYVFGGRDASRQYNGFYSFDTTTNEWKLLTPVEE--GPTPRSFHSMAADEENVYVFGGVS------ATARLNTLDS 298
o04318    VSVGSTLYTFGGRDFSRQYNGFYSFDTTTNEWKLLTPVEE--GPTPRSFHSMAADEENVYVFGGVG------AMDRIKTLDS 298
o49326    VSIGSSLYVFGGRDASRKYNGFYSFDTTKNEWKLLTPVEQ--GPTPRSFHSMTADENNVYVFGGVS------ATVRLKTLDA 299
Q39104    VAVGTKIYIFGGRDENRNFENFRSYDTVTSEWTFLTKLDEVGGPEARTFHSMASDENHVYVFGGVSKGGTMNTPTRFRTIEA 163
ESP       VTVGTK                            FLTKLDEVGGPEARTFHSMAWDDNHVYVFGGVSK           TIEA
          *::*:                               ** :::  **  *:****: *:: ********            *::

o04317    YNIVDKKWFHCSTPGDSLTARGGAGLEVVQGKVWVVYGFNG---------CEVDDVHYYDPVQDKWTQVETFGVRPSERSVF 339
o04318    YNIVDKTWFHCSNPGDSFSIRGGAGLEVVQGKVWIVYGFNG---------CEVDDVHFYDPAEDKWTQVETFGVKPNERSVF 339
o49326    YNIVDHKWVQCSTPGGSCSVRGGAGLEVVQGKVWVVYGFNG---------CEVDDVHCYDPAQDKWTQVETFGEKPCARSVF 340
Q39104    YNIADGKWAQLPDPGDNFEKRGGAGFAVVQGKIWVVYGFATSIVPGGKDDYESNAVQFYDPASKKWTEVETTGAKPSARSVF 204
ESP       YNIAEGKWIQLPDPGEQLARR            IWVVYGFATSPV
          *** : :*     **:    *             :*:****

o04317    ASAAIGKHIVIFGGEIAMDPLAHVGPGQLTDGTFALDTETLQWERLDKFGGEEETPSS-RGWTASTTATIDGKKGLVMHGGK 429
o04318    ASAAIGKHIVIFGGEIAMDPRAHVGPGQLIDGTFALDTETLQWERLDKFEG---TPSS-RGWTASTTGTIDGKKGLVMHGGK 426
o49326    ASAVVGKHILVFGGEIAMDPKAHEGPGQLSGGTFALDTETLKWEKLDKLGEEEETPSI-RGWSASTTGTIDGKKGLVMFGGK 430
Q39104    AHAVVGKYIIIFAGEVWPDLNGHYGPGTLSNEGYALDTETLVWEKLGEEG----APAIPRGWTAYTAATVDGKNGLLMHGGK 300
ESP              YILIFGGELWPDPRAHLGPGTLSDEGYALDTETLVXER                                KGLLMHGGK
                  *::*:**:  *  :* *** *    :******  :*:                                :**:*:***

o04317    APTNDRFDDLFFYGIDSA 470
o04318    APTNDRFDDLFFYGIDSV 467
o49326    AQTNDRFGDLFFYGVDSA 471
Q39104    LPTNERTDDLYFYAVNSA 341
ESP       LPTN
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Fig. 1. Alignment of the amino acid sequences of Q39104 (hypothetical 37 kDa protein) and three jasmonate-inducible proteins (named after their accession number in the TrEMBL data bank) from *Arabidopsis thaliana* and of ESP tryptic peptides. '*' indicates residues strictly conserved in all five sequences and ':' indicates residues conserved or conservatively substituted in at least four sequences.

Table 1
Purification of ESP from *Brassica napus* seeds

	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	75	0.013	100	1
Dialyzed extract	71	0.04	95	3
Q-Sepharose F.F.	69	0.25	92	19
Q-Sepharose H.P.	49	0.4	65	31
Superdex 200 HR 10/30	10	2.5	13	192

peak of about the same molecular weight. For this reason, we assumed that it was a monomer. It is interesting to note that the last step of purification caused a strong decrease of the total recovered ESP units (Table 1). This result is likely due to the complete separation of MYR and ESP, which were associated and co-eluted in the previous steps of the isolation procedure. The protein (2.5 U/mg) maintained its activity for about 45 days when stored at 4°C. Finally, using *epi*-progoitrin as a substrate, the optimum pH of ESP was around 6. This value is practically the same as that observed by Tookey for ESP of crambe [5].

3.2. Partial amino acid sequence

In order to verify whether ESP activity is associated with any protein whose primary structure is already available, we undertook a partial characterisation of the primary ESP structure. The protein was N-terminally blocked. Fig. 1 shows the amino acid sequence of all of the peptides characterised following tryptic digestion and HPLC separation, starting from 10 nmol of carbamidomethylated protein. The ESP peptides (comprising 181 residues, accounting for about 50% of the protein, as judged from the molecular weight) were aligned with those proteins with significant identities, obtained for more than one peptide with a computer search using the BLAST and FASTA3 programs [13,14]. The identity between ESP sequence and the corresponding regions of the hypothetical 37 kDa protein from *Arabidopsis* (accession code Q39104 in the TrEMBL data bank) [15] is 82% (which increases to 86% similarity taking in account conservative substitutions). In addition, all of the ESP peptides have a corresponding region in Q39104 and most of them start from a basic residue in Q39104. It can thus be concluded that ESP and Q39104 are the same protein from two different species. The computer search also revealed extensive similarities with the C-terminal portion of a number of jasmonate-inducible proteins from *Arabidopsis* (Fig. 1), none of which, however, have been characterised at the protein level. This observation links the ESP protein to jasmonate and, therefore, there is more evidence that ESP–MYR–GLs is a defence-related system in Brassicaceae.

Finally, it should be pointed out that the amino acid sequence of some ESP peptides shows some similarity with the primary structure of several myrosinase-binding proteins which have been sequenced ([16] and references therein] and other jasmonate-binding proteins [17]. Nevertheless, this similarity does not extend to the whole structure, ruling out the possibility that ESP activity is associated with such proteins.

4. Conclusions

This paper reports the first purification protocol which allows the isolation of ESP in an homogeneous form. The partial characterisation of ESP has made it possible to establish that it is the protein in *Brassica napus* which corresponds to the hypothetical 37 kDa protein (Q39104) of *Arabidopsis* and that it is related to jasmonate-inducible proteins from the same source. This finding is of high biochemical and biological importance in the comprehension of the disease resistance mechanism in cruciferous plants, where the MYR–GLs system is present.

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References

[1] Ettlinger, M.G. and Kjaer, A. (1968) Recent Adv. Phytochem. 1, 59–144.
[2] Sørensen, H. (1990) in: Canola and Rapeseed, Production, Chemistry, Nutrition and Processing Technology (Fereidoon Shahidi Ed.), pp. 149–172. Van Nostrand Reinhold, New York.
[3] Chew, F.S. (1988) in: Biologically active natural products, ACS Symposium Series, 380, pp. 155–181.
[4] Louda, S. and Mole, S. (1991) in: Herbivore, their Interaction with Secondary Plant Metabolites, 2nd edn., Vol. 1, pp. 123–164. Academic Press, San Diego, CA.
[5] Tookey, H.L. (1973) Can. J. Biochem. 51, 1305–1310.
[6] Petrosky, R.J. and Tookey, H.L. (1982) Phytochemistry 21, 1903–1905.
[7] MacLeod, A.J. and Rossiter, J.T. (1985) Phytochemistry 24, 1895–1898.
[8] Visentin, M., Tava, A., Iori, R. and Palmieri, S. (1992) J. Agric. Food Chem. 40, 1687–1691.
[9] Leoni, O., Carli, C., Bernardi, R. and Palmieri, S. (1999) 10th International Rapeseed Congress.
[10] Negri, A., Ceciliani, F., Tedeschi, G., Simonic, T. and Ronchi, S. (1992) J. Biol. Chem. 267, 11865–11871.
[11] Bradford, M.M. (1976) Anal. Biochem. 72, 255–260.
[12] Wallig, M.A., Gould, D.H., Fettman, M.J. and Willhite, C.C. (1988) Food Chem. Toxicol. 26, 149–157.
[13] Atschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. (1997) Nucleic Acids Res. 25, 3389–3402.
[14] Pearson, W.R. and Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[15] Quinley, F.R. (1993) Submitted to the EMBL/GenBank/DDBJ data bank.
[16] Taipalensuu, J., Eriksson, S. and Rask, L. (1997) Eur. J. Biochem. 250, 680.
[17] Geshi, N. and Brandt, A. (1998) Planta 204, 295–304.