



A contaminant trypsin-like activity from the timothy grass pollen is responsible for the conflicting enzymatic behavior of the major allergen Phl p 1

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ABSTRACT

We intend to solve whether or not Phl p 1 can be regarded as a protease. A group reported that Phl p 1 has papain-like properties and later on, that this allergen resembles cathepsin B, while another one demonstrated that Phl p 1 lacks proteinase activity and suggested that the measured activity may rise either from a recombinant Phl p 1 contaminant or as a result of an incompletely purified natural allergen. A third group reported Phl p 1 to act by a non-proteolytic activity mechanism. We report the purification of the natural Phl p 1 by means of hydrophobic interaction, gel filtration and STI-Sepharose affinity chromatographies. The Phl p 1 purity was assessed by silver-stained SDS-PAGE and by 'in-gel' and 'gel-free' approaches associated to mass spectrometry analyses. The proteolytic activity was measured using Boc-Gln-Ala-Arg-AMC and Z-Phe-Arg-AMC as substrates. While amidolytic activity could be measured with Phl p 1 after rechromatography on gel filtration, it however completely disappeared after chromatography on STI-Sepharose. The contaminant activity co-eluting with Phl p 1 was not affected by cysteine proteases inhibitors and other thiol-blocking agents, by metalloproteases inhibitors and by aspartic proteases inhibitors. However, it was completely inhibited by low molecular weight and proteinaceous serine proteases inhibitors. TLCK, but not TPCK, inhibited the contaminant activity, showing a trypsin-like behavior. The pH and temperature optimum were 8.0 and 37 °C, respectively. These data indicated that Phl p 1 is not a protease. The contaminant trypsin-like activity should be considered when Phl p 1 allergenicity is emphasized.

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1. Introduction

The ability of certain proteins to induce an allergic response in susceptible individuals is well established. Symptoms can range from mild erythema or rhinitis, to acute and possibly fatal, anaphylactic shock. Because such allergic responses require complex interactions between proteins and the immune system, they are notoriously difficult to predict. Nevertheless, it is clear that some proteins are intrinsically more allergenic than others. The challenge is to identify those characteristics that confer to proteins the potential to induce allergic sensitization and allergic disease. It is now well established that one should first consider the individual epitopes within proteins that can be recognized by the immune system and that are fundamental requirements for all immune responses. However, it seems that the presence of appropriate epitopes alone is not sufficient to confer an allergenic potential to a given protein; other features and properties have to be considered. One should consider for example, the effects that resistance to proteolysis, post-translational modifications, and enzymatic activity may confer [1–3].

Allergens possess a wide range of biologic activities. For some, mechanisms have been identified whereby the activity could contribute

directly to the efficiency of the allergen. Indeed, it has been shown that the protease activity of several allergens is essential for induction of Th2 and IgE responses [4–6]. Indeed, the cysteine protease allergen, Der p 1, has been shown to influence the activity of dendritic cells, T cells and B cells in vitro [4,7]. It has also shown that protease allergens have immunologically relevant consequences in vivo such as the production of Th2-promoting cytokines and the induction of Th2 differentiation [8].

Proteases from a number of airborne allergenic sources have been purified [9–14]. The aeroallergens derived from plant pollens seem to be important triggers for asthma and rhinitis and interestingly, many allergenic types of pollen have been reported to present proteolytic activity [12,15–17]. Scarce informations about whether purified allergenic proteins of pollens have intrinsic protease activity are however available [18–20]. It is commonly recognized that this activity might for instance disrupt the epithelial barrier, to promote sensitisation, not only to proteases but also to other released pollen proteins without intrinsic enzymatic activity [11].

Pollens contain a variety of allergens, including those of group 1 which are worldwide, among the most important mediators of type 1 allergic reactions [21,22]. These allergens belong to the β -expansin family and act as mediators of cell-wall loosening, an essential process for the plant growth. These are glycoproteins of about 30 kDa, with a carbohydrate content of about 5%. They are located on the surface of

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pollen grains and are quickly released upon hydration [18]. Therefore, they readily dissolve in the mucous membrane of human respiratory epithelia. The timothy grass pollen allergen, Phl p 1, can be used as a model for group 1 allergens, since it contains most of the B-cells as well as T-cells epitopes displayed by this group [18,20].

To date, there are conflicting reports about the proteolytic activity of Phl p 1. Grobe et al. [18,20] have proposed that this highly immunogenic allergen is a protease. Phl p 1 expressed in *Pichia pastoris*, along with the natural allergen purified by affinity chromatography (on an antibody-immobilized column) was shown to degrade a nitroanilide substrate in a colorimetric protease assay and to cleave a casein zymogram. Proteolysis was inhibited by cysteine and serine proteases inhibitors but not by metallo or aspartic proteases inhibitors [18]. It was also shown that Phl p 1 could be aligned with three functionally important motifs, including active-site cysteine and histidine residues that define the CA1 papain-like cysteine proteases [18]. Further investigation, by the same group, demonstrated that β -expansins exhibit sequence similarity with the cathepsin B cysteine protease [20]. Similar to CA1 proteinases, β -expansins are secreted and exported to the cell walls in an inactive proform share a similar molecular mass and an optimum functional pH, and require auto-activation by reducing agents. The authors conclude that the intrinsic proteolytic activity associated to β -expansins may mediate plant cell wall loosening and, theoretically promote an increased permeability of the airway epithelium [20]. These findings, however, have been directly contested by Li and Cosgrove [19]. In a series of experiments, three purified native group 1 allergens from timothy grass, rye grass, and maize were studied. Results from (i) a colorimetric protease assay, (ii) a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), (iii) a native PAGE zymography and digestion of casein-containing agarose gels, all demonstrated a lack of proteolytic activity [19]. By resolving the 3D structure of Zea m 1 (a Phl p 1 homologue from *Zea mays*), Yennawar et al. [23] concluded from their crystallographic model that Zea m 1 has no resemblance to a papain-like fold. Very recently [24], Petersen's group, emphasizing the association of an enzymatic activity with Phl p 1 [18,20], reported the ability of Phl p 1 to activate respiratory epithelial cells by a non-protease mechanism.

In this present study, we intended to solve whether Phl p 1 can be regarded or not as a protease by considering the naturally purified allergen. The protease activity was assessed using highly sensitive fluorogenic substrates and different classes of protease inhibitors, both on a Phl p 1

preparation obtained after two successive gel filtration chromatographies and on a preparation obtained after a further affinity chromatography using soybean trypsin inhibitor (STI) as immobilized ligand. The purity of the allergen was controlled by silver-stained SDS-PAGE and by mass spectrometry analyses.

2. Results

2.1. Purification of Phl p 1

Fractionation of a timothy grass pollen extract using hydrophobic interaction chromatography resulted in two flow-through fractions (peaks I and II in Fig. 1) and in a fraction eluted in water (peak III in x. 1). SDS-PAGE analysis confirms the presence of Phl p 1 in the first flow through fraction by protein bands with apparent molecular weights of approximately 29–32 kDa corresponding to Phl p 1 isoforms, and by other high and low molecular weight contaminant bands (line 2 in the inset of Fig. 1). A further fractionation of this fraction by size exclusion chromatography (Fig. 2) followed by rechromatography of the pool containing the 29–32 kDa double band (indicated by the arrow in Fig. 2) led to a sharp and symmetrical chromatogram (Fig. 3) associated to an homogeneous Phl p 1 fraction (line 2 in the inset 1 of Fig. 3). The 29–32 kDa bands were indeed identified as Phl p 1 isoforms [25,26] while the low molecular weight protein bands might correspond to the Phl p 2/3 group allergen [29]. The second flow-through fraction (peak II) was free of protein material but contained yellow-brown-colored compounds. Peak III is made up of protein material with different molecular weights that were not further analyzed here.

2.2. Enzymatic activity towards peptide substrates

Typical fluorescence profiles versus time are shown in the Inset 2 of Fig. 3. Both substrates, Boc-Gln-Ala-Arg-AMC and Z-Phe-Arg-AMC, were cleaved by the Phl p 1 pool obtained after rechromatography on Superdex 75. However, using the same final substrate concentration, the relative amidase activity in the presence of Boc-Gln-Ala-Arg-AMC, a good trypsin substrate [27,28] was found much higher when compared to that obtained with Z-Phe-Arg-AMC. According to these data, the further experiments were conducted with the former substrate.

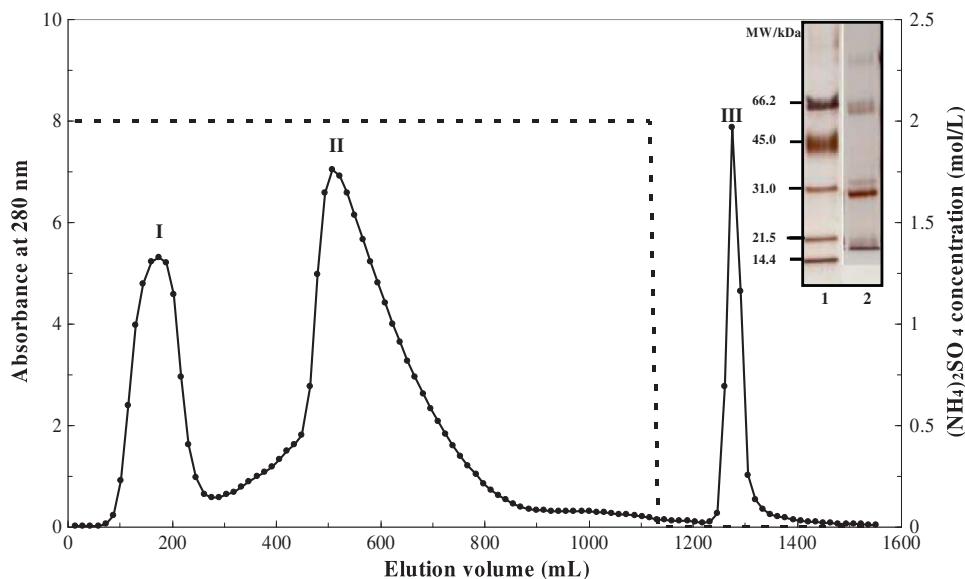


Fig. 1. Phl p 1 purification. Hydrophobic interaction chromatography on Phenyl Sepharose of the timothy grass pollen extract. The flow-through fraction (peaks I and II) and retained fraction (III) were collected. *Inset:* SDS-PAGE analysis. Line 1, protein molecular weight markers and line 2, the flow-through fraction I.

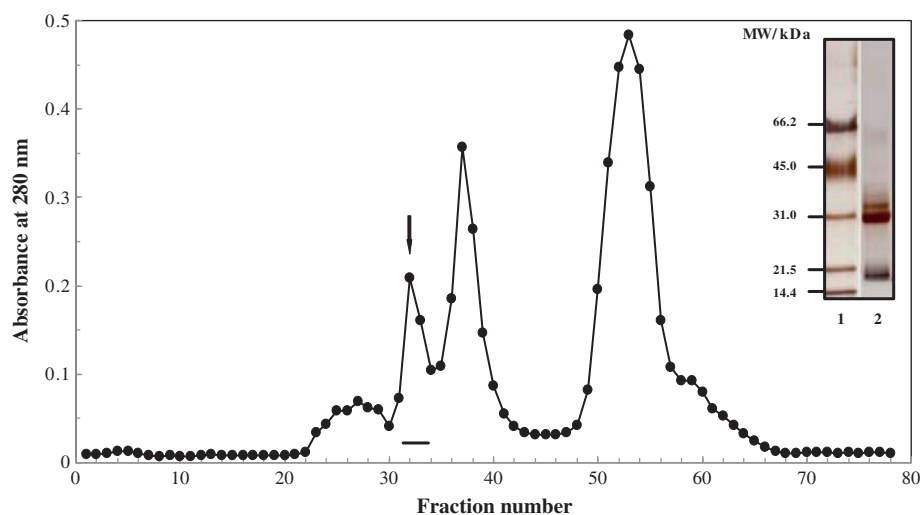


Fig. 2. Gel filtration chromatography on Superdex 75 of peak I obtained after hydrophobic interaction chromatography. *Inset:* SDS-PAGE analysis. Line 1, protein molecular weight markers and line 2, protein peak indicated by the arrow containing Phl p 1.

2.3. Inhibitory activity measurements

As shown in Fig. 4, the amidase activity of the Phl p 1 sample obtained after a second Superdex 75 run was completely inhibited in the presence of serine protease inhibitors, such as Pefabloc SC, PMSF, benzamidine, soybean trypsin inhibitor (STI) and papaya protease inhibitor (PPI). No inhibitory effect could however be observed

with pepstatin A, an aspartic protease inhibitor, EDTA, a metallo protease and E-64, iodoacetic acid, iodoacetamide and HgCl_2 , all cysteine proteases inhibitors. All these data are in favor of a serine-like protease activity.

In addition, the enzymatic activity of the Phl p 1 pool was also performed on all the chromatographic fractions contained in the peak resulting from the rechromatography on Superdex. As shown in

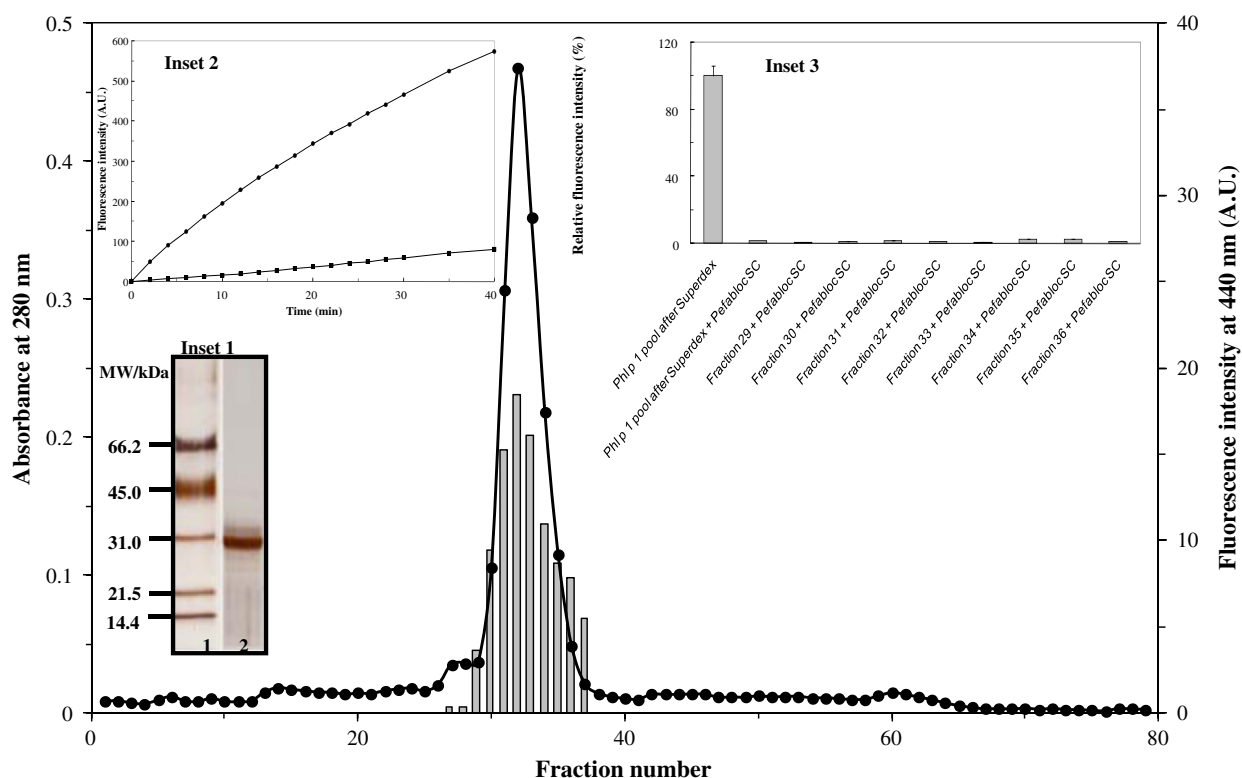


Fig. 3. Rechromatography of the Phl p 1-containing fraction on Superdex 75. Histograms presentation corresponds to enzymatic activities of the selected chromatographic fractions. *Inset 1:* SDS-PAGE analysis. Line 1, protein molecular weight markers and line 2, Phl p 1 after rechromatography on Superdex 75. *Inset 2:* Fluorimetric detection of enzymatic activity of Phl p 1 pool, obtained after rechromatography on Superdex 75, towards the substrates Z-Phe-Arg-7-amido-4-methylcoumarin (■) or Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin (●) in a 50 mM Tris/HCl buffer at pH 8.0 containing 3 mM EDTA and 0.01% Brij 35. More details were described in Materials and Methods section. *Inset 3:* Amidase activity of Phl p 1 pool obtained after rechromatography on Superdex 75 and of chromatographic fractions constituting the peak, in the absence and presence of Pefabloc SC. The assays were carried out in triplicate. More details were described in Materials and Methods.

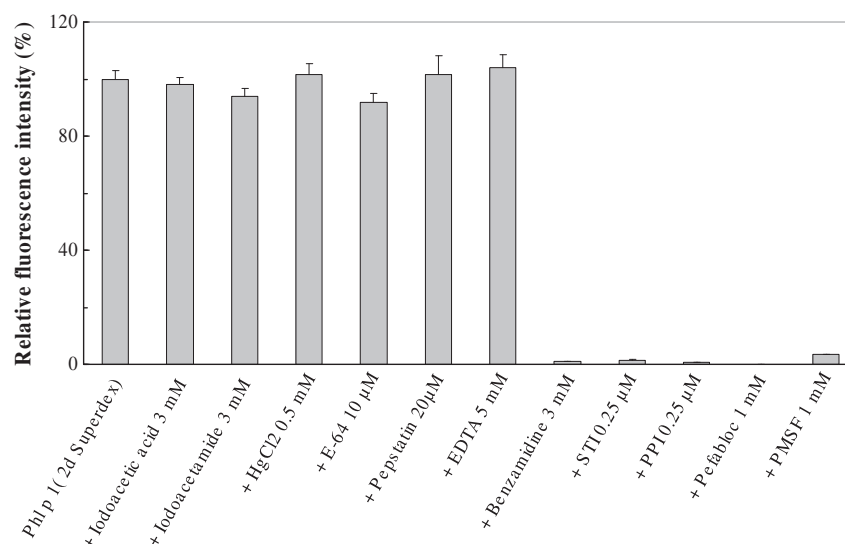


Fig. 4. Amidase activity of the Phl p 1 pool sample obtained after rechromatography on Superdex in the absence and presence of different proteases inhibitors. The assays were carried out in triplicate. More details were described in [Materials and Methods](#).

[Fig. 3](#), the enzymatic activity can be detected in all the fractions constituting the peak, demonstrating at this purification step that Phl p 1 and the contaminant with protease activity co-eluted simultaneously. This activity was completely inhibited in the presence of Pefabloc SC (inset 3 of [Fig. 3](#)) but remained unaffected in the presence of E-64 (not shown).

2.4. Mass spectrometry identification and characterization

Assuming a serine-like protease activity associated to the Phl p 1 sample we charged it onto a STI-Sepharose column. The chromatogram ([Fig. 5](#)) revealed the presence of a flow through fraction (UF) with a double protein band characterizing the two Phl p 1 isoforms (line 2 in the inset 1 of [Fig. 5](#)) and of a specifically retained fraction (abbreviated here STI-R PF for STI-Retained Protein Fraction) that was eluted with a 3 mM HCl solution.

No amidolytic activity was detected with the affinity-purified Phl p 1 (inset 2 of [Fig. 5](#)). Amidolytic activity was however well detected in the protein fraction specifically fixed on the affinity gel media and eluted with 3 mM HCl (inset 2 of [Fig. 5](#)). These results clearly indicate that the protease activity can only be separated from Phl p 1 after an affinity chromatography, that demonstrated their co-elution on the hydrophobic and size exclusion gel media.

Inhibitory measurements performed on the STI-R PF fraction confirmed a serine-like protease behavior, being specifically and exclusively affected by serine proteases inhibitors. To further assess its trypsin-like or chymotrypsin-like property, TLCK and TPCK were used. As we can see from [Fig. 7](#), STI-R PF presented a trypsin-like activity since slightly affected by TPCK while totally inhibited by TLCK.

The enzymatic activity of STI-R PF towards Boc-Gln-Ala-Arg-AMC was optimum around pH 8.0 ([Fig. 8A](#)). In a MOPS buffer (pH 6.6), the activity was only 25% of the optimum value and a weakly

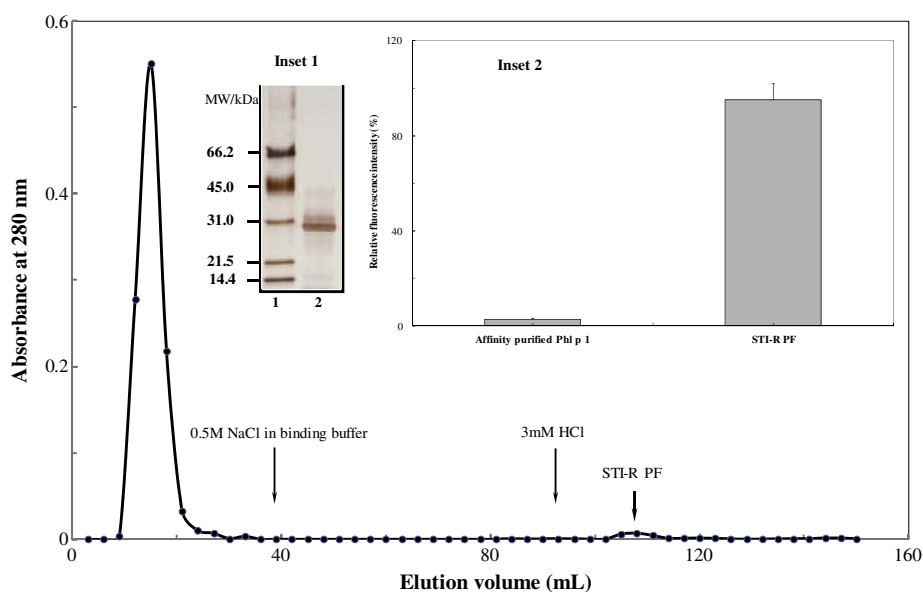


Fig. 5. Affinity chromatography of Phl p 1 on STI-Sepharose. UF: unretained fraction; STI-R PF: STI-Retained Protein Fraction. More details were described in [Materials and Methods](#). Inset 1: SDS-PAGE analysis. Line 1, protein molecular weight markers and line 2, unretained fraction. More details were described in [Materials and Methods](#). Inset 2: Amidase activity of affinity-purified Phl p 1 and of the protein fraction specifically retained on STI-Sepharose (STI-R PF). The assays were carried out in triplicate. More details were described in [Materials and Methods](#).



B

Heavy Band

Pollen allergen Phl p 1 (Phleum pratense) : gi :1171008

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1 MASSSSVLLV VVLFVAVFLGS AYGIPIKVPPG PNITATYGDK WLDAKSTWYG
51 KPTGAGPKDN GGACGYKDVD KPPFSGMTGC GNTPIFKSGR GCGSCFEIKC
101 TKPEACSGEP VVVHITDDNE EPIAPYHFDL SGHAFGAMAK KGDEQKL RSA
151 GELEIQFRRV KCKYPEGTVK TFHVEKGSNP NYLALLVKYV NGDGDVVAVD
201 IKEKGKDKWI ELKESWGAIW RIDTPDKLTG PFTVRYTTEG GTKTEADVI
251 PEGWKADTSY ESK

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Start - End	Observed	Mr(calc)	Delta	Miss	Sequence
46 - 58	1349.6633	1348.6775	-0.0215	0	K.STWYGKPTGAGPK.D
68 - 87	2167.9729	2167.0078	-0.0421	0	K.DVDKPPFSGMTGCGNTPFK.S
91 - 99	1057.4437	1056.4368	-0.0004	0	R.GCGSCFEIK.C
149 - 158	1149.5819	1148.5825	-0.0079	0	R.SAGELEIQF.R
149 - 159	1305.6799	1304.6836	-0.0110	1	R.SAGELEIQFRR.V
177 - 188	1288.6980	1287.7186	-0.0279	0	K.GSNPNYLALLVK.Y
214 - 221	1004.5053	1003.4875	0.0105	0	K.ESWGAIW.I
222 - 235	1559.8312	1558.8355	-0.0116	1	R.IDTPDKLTGPFTVR.Y
236 - 255	2210.9946	2210.0378	-0.0505	0	R.YTTEGGTKTEADVIPEGWK

Light Band

Pollen allergen Phl p 1 (Phleum pratense) : gi :3901094

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1 MASSSSVLLV VALFVAVFLGS AHGIPKVPPG PNITATYGDK WLDAKSTWYG
51 KPTAAGPKDN GGACGYKDVD KPPFSGMTGC GNTPIFKSGR GCGSCFEIKC
101 TKPEACSGEP VVVHITDDNE EPIAAYHFDL SGIAFGSMAM KGDEQKL RSA
151 GEVEIQFRRV KCKYPEGTVK TFHVEKGSNP NYLALLVKFV AGDGDVVAVD
201 IKEKGKDKWI ALKESWGAIW RIDTPEVLKG PFTVRYTTEG GTKGEAKDVI
251 PEGWKADTAY ESK

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Start - End	Observed	Mr(calc)	Delta	Miss	Sequence
46 - 58	1363.6626	1362.6932	-0.0378	0	K.STWYGKPTAAGPK.D
68 - 87	2167.9729	2167.0078	-0.0421	0	K.DVDKPPFSGMTGCGNTPFK.S
91 - 99	1057.5052	1056.4368	0.0611	0	R.GCGSCFEIK.C
147 - 158	1404.8051	1403.7520	0.0458	1	K.LRSAGEVEIQF.R
149 - 158	1135.6360	1134.5669	0.0618	0	R.SAGEVEIQF.R
177 - 188	1288.7972	1287.7186	0.0713	0	K.GSNPNYLALLVK.F
214 - 221	1004.5665	1003.4875	0.0717	0	K.ESWGAIW.I
222 - 235	1571.9805	1570.8718	0.1014	1	R.IDTPEVLKGPFTVR.Y
248 - 255	943.5533	942.4810	0.0650	0	K.DVIPEGWK.A

Fig. 6. Identification and characterization of Phl p 1 by mass spectrometry. A: MALDI-TOF analysis of tryptic peptides from heavy and light bands detected in SDS-PAGE of affinity-purified Phl p 1 (line 2 in Inset 1 of Fig. 5). B: sequences and matched peptides (* or o) of both different isoallergens of Phl p 1 (NCBI numbers gi /1171008 and gi /3901094) detected in the heavy and light SDS-PAGE bands, respectively.

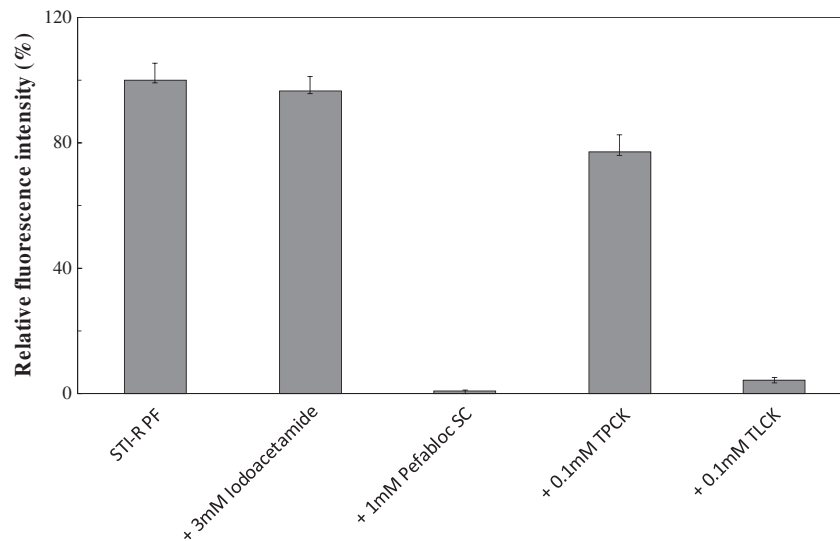


Fig. 7. Amidase activity of the fraction specifically retained on STI-Sepharose (STI-R PF) in the presence of different proteases inhibitors. The assays were carried out in triplicate.

detectable activity was observed below pH 5.5. The optimum temperature of Phl p 1 was found around 37 °C (Fig. 8B).

Using the peptide mass finger print analysis obtained by MALDI-TOF/MS, the affinity-purified 29–32 kDa double band was confirmed to be Phl p 1 (Fig. 6). Interestingly, each SDS-PAGE band (heavy and light) corresponds to a different Phl p1 isoallergen characterized by

distinct molecular weights (MW_{th} of mature forms: 26157 Da and 25957 Da (Fig. 6). The apparent high molecular weight observed on SDS-PAGE is the result of glycosylation. Except for the isoforms of Phl p 1, no other protein contaminant could be detected by LC-MS/MS analyses of the same affinity-purified Phl p 1 sample (not shown).

Interestingly, mass spectrometry analysis using a new high resolution triple ToF 5600 (ABSciex) of the protein fraction specifically retained on STI-Sepharose column (STI-R PF) unambiguously showed the presence in this fraction of a serine-like protease identified from 3 sequenced peptides (Fig. 9). Indeed, the identified serine protease presented homology to an uncharacterized protein from Glycine max (C6TKC9_SOYBN) and showed a high homology (70–90% of identity) with a lot of serine proteases.

3. Discussion

Grass pollen represents one of the most potent and frequent elicitors of respiratory allergies [29]. Until now, eleven different allergens have been characterized on the basis of their biochemical, immunological and structural properties [22,29]. IgE binding studies realized in different populations of grass pollen allergic patients, as well as studies evaluating their in vitro and in vivo allergic activity, indicate that four major timothy grass pollen allergens (Phl p 1, Phl p 2, Phl p 5 and Phl p 6) represent a major part of its allergenic activity [30–36]. Later on, two additional timothy grass pollen allergens, Phl p 4 and Phl p 13, representing the high molecular mass allergen fraction (50–60 kDa), were also described as major allergens according to IgE binding frequencies [26,36,37]. According to the I.U.I.S. nomenclature (<http://www.allergen.org>), nine allergens were described for Phleum pratense (Phl p 1, 2, 4, 5, 6, 7, 11, 12 and 13).

Although it is established that asthma and seasonal rhinitis/conjunctivitis complications are related in the majority of cases to airborne allergens sensitisation [38,39], the mechanism by which exposure to these allergens triggers an allergic response remains incompletely understood. It is however supposed that the biological function of an allergen might be an important factor for its allergenicity. From these biological activities, proteolytic activity of allergen sources has been one of the functions proposed to lead to the enhancement of allergenicity [4,40–42].

In the present study the major grass pollen allergen, Phl p 1, which is recognized by up to 95% of the allergic individuals [43] was investigated for its protease activity. The allergen was purified from its natural source to obtain the natural glycosylated protein form. It was indeed

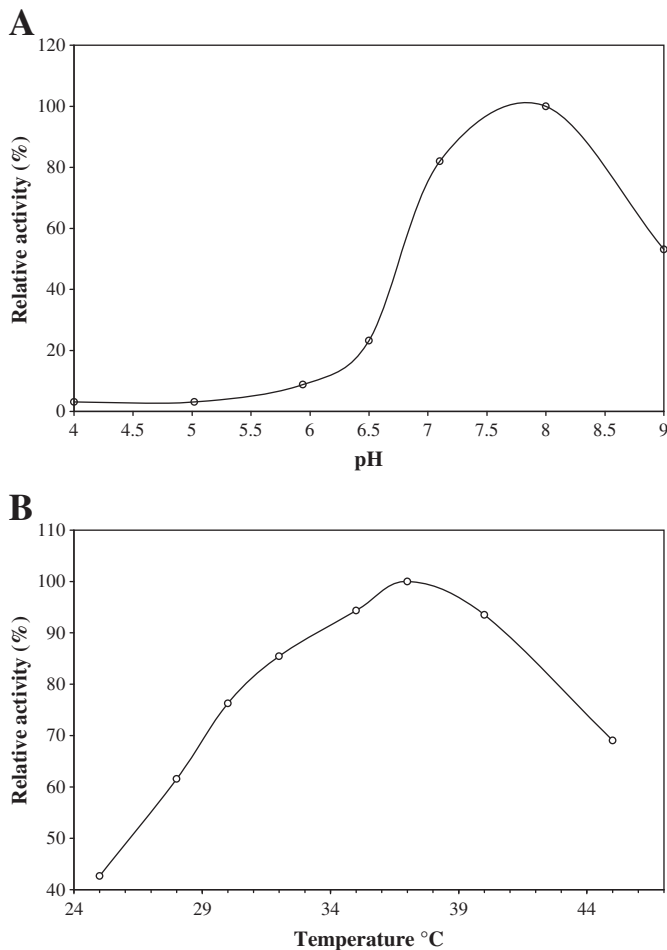


Fig. 8. pH (A) and temperature (B) optimum of the protein fraction specifically retained on STI-Sepharose (STI-R PF). For more details, see Materials and Methods.

reported that the Phl p 1 recombinant obtained in an eukaryotic expression system was hyperglycosylated, with up to 15% of glycan mass [44]. Interestingly, the glycan moiety located in the N-terminal portion and which accounts for ~5% of the protein mass of the natural allergen was reported to influence the IgE reactivity of Phl p 1 [45]. SDS-PAGE analysis after silver staining of the Phl p 1 sample obtained, after rechromatography on Superdex 75 and affinity chromatography, revealed the presence of a typical double band in the 29–32 kDa apparent molecular weight range. MALDI-TOF mass spectrometry after ‘in-gel’ tryptic digestion was used to identify this affinity purified double protein bands. Each SDS-PAGE band (heavy and light) corresponds to a different Phl p 1 isoallergen with two distinct molecular weights: 26157 Da and 25957 Da (Fig. 6). Except for these two isoforms of Phl p 1, no other component can be detected in these two selected bands. This heterogeneity in size of the natural Phl p 1 sample has already been described by Petersen et al. [25,46]. Using antibody-immobilized affinity chromatography, Grobe et al. [18] noticed the presence of three bands in their purified natural Phl p 1, all of them could be identified, by monoclonal and IgE antibodies, as isoforms or degradation products of Phl p 1. To have the guarantee that the affinity-purified Phl p 1 was free of any contaminating protease activity (in addition to the absence of any detectable enzymatic activity), the same Phl p 1 sample solution as deposited on SDS-PAGE (line 2 of the inset 1 of Fig. 5) was further analyzed by LC-MS/MS after ‘in-solution’ tryptic digestion.

Interestingly, no other protein except Phl p 1 could be detected in the purified Phl p 1 (not shown). Interestingly, a gel-free analysis of the STI-P RF fraction emphasized unambiguously the presence of a serine-like protease. One interesting aspect of the Phl p 1 and its tightly associated ‘extrinsic’ protease activity is the possibility that such activity could be related to the high prevalence of individuals allergic to group I allergens. Therefore, it can be reasonably proposed that by enabling the allergen to breach the integrity of the airway epithelial barrier, proteolytic activity might play an adjuvant pro-allergic role influencing immunogenicity. Vinhas et al. [47] demonstrated that pollen proteases compromise the airway epithelial barrier through disruption of transmembrane adhesion proteins and degradation of airway bioactive peptides. For the Der p 1 mite dust allergen, it was also reported that its potent IgE-eliciting property might be due to its cysteine protease activity. Der p 1 was shown (i) to cleave CD40 on dendritic cells resulting in decreased interleukin-12 production [7], (ii) to cleave CD23 (IgE receptor) on B cells, disrupting a negative feedback mechanism resulting in excessive IgE production [48] and (iii) to cleave CD25 (the α -subunit of the interleukin-2 receptor) on T cells, reducing proliferation and γ interferon production of T cells [49,50]. All together, the cleavage of these molecules could favor a Th2-mediated response. The cysteine proteases Der p 1 and Der f 1 have also been found to proteolytically degrade and inactivate lung surfactant proteins, which play key roles both in innate immune defense and allergy [51]. Sun et al. reported that Der p 3 and

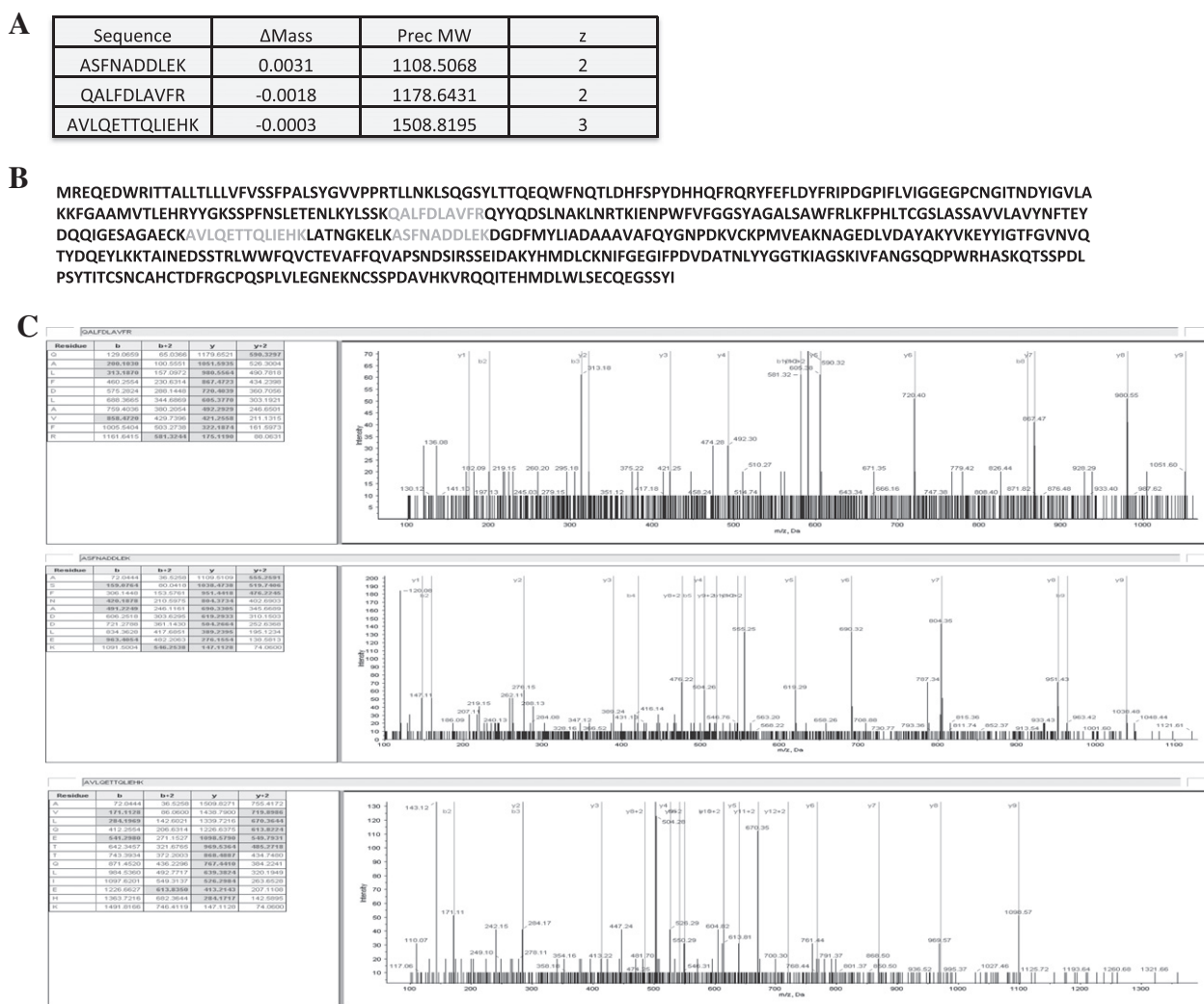


Fig. 9. Identification and characterization of a serine-like protease from the protein fraction specifically retained on STI-Sepharose chromatography (STI-R PF) using mass spectrometry. (A) Sequences and (B) matched peptides of uncharacterized protein (Glycine max) (C6TKC9_S0YBN). The sequences were determined with a 99 score confidence using ProteinPilot software (ABSciex). (C) MS/MS spectra of each matched peptide.

Der p 9, two serine proteases of house dust mite, were able to trigger protease-activated receptor-2 (PAR-2), and could explain the pro-allergic environment induced by these allergens [52]. In another study Cur 1, a fungus major allergen serine protease, was found to exacerbate airway inflammation in a murine model of asthma. This inflammation was significantly reduced by using the inactivated allergen counterpart [53]. Very recently, a novel major Japanese cedar pollen allergen was identified as an aspartic protease [54].

Although the precise biological function(s) of the major allergens of timothy grass pollen is(are) still unknown, there are some nice reports on other homologous proteins. A clue to their biological function was first obtained, when a sequence similarity to expansins was noted [55]. Subsequently the allergen from maize pollen, Zea m 1, was also reported to exhibit expansin activity specifically towards grass cell walls [56]. Expansins, subdivided into α - and β -expansins, are described as being key catalysts of cell wall loosening, a process necessary for plant cell growth, cell separation and other related processes [56,57]. The involvement of expansins in growth, survival and/or reproduction of grasses is also presumed [58].

In the case of Phl p 1, there are controversial opinions regarding its protease activity. In a first report by Grobe et al. [18], Phl p 1 was proposed as a novel papain-related protease on the basis of sequence comparison analysis and on proteolytic and inhibitory activities. These authors suggested that the implication of the reported papain activity of Phl p 1 might consist in the grass cell walls, in degrading specific structural target proteins, namely the extensins (hydroxyproline-rich glycoproteins), contributing in that way to the cell wall extension mechanism. The identity of Phl p 1 as a cysteine protease, albeit with unusual properties, was further demonstrated using several classes of proteases inhibitors [18]. Surprisingly, these authors reported that E-64, a highly specific and potent cysteine protease inhibitor, had no effect. On the contrary, Pefabloc SC, a specific serine protease inhibitor reacted with Phl p 1. The authors attributed this behavior to the deletion/mutation of some key amino acids implicated in the activity process [18]. It is not clear however, if the authors conducted their inhibitory studies both with the recombinant allergen and with its natural counterpart.

In contrast to this finding, another study by Li and Cosgrove [19] concluded that Phl p 1, both recombinant and natural forms, lack protease activity towards a variety of chromogenic substrates and a variety of zymogram systems, and consequently does not cause wall loosening (as a result of proteolytic action). The authors also tested the effect of the protease inhibitors used by Grobe et al. [18] on the wall extension activity to check if the idea that β -expansins have proteolytic activity was correct. Their results demonstrated a slight or even no effect on the β -expansin activity. By resolving the 3D structure of a Phl p 1 homologue, Zea m 1 (or EXPB1), Yennawar et al. [23] concluded that there is no correspondence between Zea m 1 fold and a papain-like fold. Furthermore, the residues implicated in the catalytic activity of papain-like proteases are not conserved in Zea m 1 structure. For Li and Cosgrove [19], the protease activity described for the recombinant Phl p 1 reported by Grobe et al. [18] was likely due to a trace contamination by a protease present in the expression medium, or to the natural Phl p 1 that may have been incompletely separated from a highly active protease. By using a native PAGE zymogram, Li and Cosgrove detected an active diffused band with the crude grass pollen extract, physically separated from group I allergens, while no activity could be detected, on the same zymogram, with the purified natural Phl p 1 [19]. In the absence of any intrinsic protease activity of Phl p 1, Li and Cosgrove suggested that cell walls loosening by β -expansins was most likely the result of a polysaccharide-polysaccharide interactions weakening [19].

In order to get more insights into this controversial finding, Pöppelmann et al. [59] tested whether the described Phl p 1 protease activity was due to the recombinant allergen or to irrelevant contaminants of the yeast expression system. They made use of zymogram

techniques with different protein substrates at different concentrations. By combination of immunoblotting, zymography and different class of specific proteases inhibitors, this group demonstrated that both, recombinant and natural Phl p 1, have no protease activity, even when incubated for 15 hours. Only the Phl p1-depleted yeast supernatant showed proteolytic activity associated with a 50 kDa protein band. Detailed analysis of this yeast enzyme, by zymography inhibitory tests, demonstrated its complete inhibition with Pefabloc SC and a strong inhibition with PMSF. On the basis of this inhibition pattern, the authors classified the enzyme as a serine protease.

In our study, we were also unable to detect any enzymatic activity based on colorimetric assays. Indeed, a natural Phl p 1 sample obtained after rechromatography on Superdex 75 was not able to cleave two synthetic substrates: *N*- α -benzoyl-DL-arginine-p-nitroanilide and Boc-Ala-Ala-Gly-p-nitroanilide, even after an incubation period of 24 hours at 37 °C (not shown). This same Phl p 1 sample was however able to efficiently cleave fluorogenic peptide-based substrates.

In a recent work, Röschmann et al. [24] reported that Phl p 1 was supposed to activate respiratory epithelial cells by a non-protease mechanism. These authors found that while Phl p 1 could interact with respiratory epithelial cells and subsequently release allergic mediators (like IL-6 and IL-8), it was found unable to exert proteolytic activity. They concluded that its activating capacity was probably driven by an indirect mechanism through which the allergen might cross the epithelial barrier and attract immunocompetent cells [24]. In the mean way, Österlund et al. [60] demonstrated that non-proteolytic aeroallergens from mites, cats and dogs exert adjuvant-like activation of bronchial epithelial cells.

4. Concluding remarks and perspectives

In the present work, by using both a much sensitive, fluorimetric based protease activity test and mass spectrometry analyses, we clearly demonstrated the presence of a contaminant serine protease activity which co-eluted with Phl p 1 on hydrophobic interactions and on two successive size-exclusion chromatographies. Further analyses of the inhibition pattern have shown that STI-R PF was completely inhibited by low molecular weight serine proteases inhibitors such as Pefabloc SC, PMSF, benzamidine and by STI and PPI, two proteinaceous serine proteases inhibitors. This enzymatic activity was found insensitive to aspartic and metallo proteases inhibitors as well as cysteine proteases inhibitors. However, it was completely inhibited by both, low molecular weight and proteinaceous serine proteinases inhibitors, assuming a serine-like protease property. Furthermore, the finding that this contaminant activity was only slightly affected by TPCK while completely inhibited by TLCK is in favor of a trypsin-like behavior. This finding is in agreement with its identification as a serine-like protease by mass spectrometry analyses.

In conclusion, our data demonstrated that Phl p 1 co-eluted with a contaminant trypsin-like protease activity that could reasonably be linked to the conflicting enzymatic activity of this pollen major allergen. Optimum pH and temperature measurements indicated a pH value of about 8.0, which is in favor of a serine-like protease mechanism [61–63] and a temperature of about 37 °C.

After affinity chromatography, both, MALDI-TOF/MS as LC-MS/MS analyses clearly identified Phl p 1 as the only molecular species present in the double band excised from the gel or found in a Phl p 1 solution. In addition, the identification of a trypsin-like protease activity specifically retained on a STI-Sepharose column clearly emphasized that the enzymatic activity measured here was exclusively associated to this contaminant protease. We would like to underline that using fluorescence spectroscopy to measure enzymatic activity remains in combination with other spectrometric and electrophoretic approaches, a reliable tool to guarantee the purity of Phl p 1 preparations. Moreover, taking into account that specific serum IgE dosages based on ELISA technique and using purified Phl p 1, 2, 4, 5, 6, 7, 11

and 12 are available to the routine laboratory (Unicap®, Pharmacia Diagnostics AB, Uppsala, Sweden), it will be interesting to check if the enzymatic activity measured here can be linked, directly or indirectly, to Phl p 1 allergenicity. This study is in progress.

5. Materials and methods

5.1. Materials

Timothy grass (*Phleum pratense*) pollen was purchased from Pharmallerga (Czech Republic). Soybean trypsin inhibitor (STI, type 1-S, lot 101K7041), EDTA, iodoacetic acid, N-p-Tosyl-L-phenylalanine chloromethyl ketone (TPCK), benzamidine hydrochloride, mercuric(II) chloride (HgCl_2) and Phenylmethanesulfonyl fluoride (PMSF) and N- α -benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA) were purchased from Sigma-Aldrich (Steinheim, Germany). Iodoacetamide was from Calbiochem (Darmstadt, Germany). 4-[(2S,3S)-3-carboxyoxiran-2-ylcarbonyl-L-leucylamido]-butylguanidine (E-64), pepstatin A and Pefabloc SC were from Roche. Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin, Z-Phe-Arg-7-amido-4-methylcoumarin and Boc-Ala-Ala-Gly-pNA were from Bachem (Darmstadt, Germany). Phenyl Sepharose 6 Fast Flow (high sub), Superdex 75, NHS-activated Sepharose 4B, precast SDS-PAGE gels (ExcelGels, gradient 8–18%) kit and protein silver staining kit for routine SDS-PAGE were from GE-Healthcare (Uppsala, Sweden). Molecular mass standards and compatible mass spectrometry protein sequencing silver staining kit were purchased from Bio-Rad Laboratories (Hercules California, SA). Papaya proteinase inhibitor (PPI) was purified from the soluble fraction of papaya (*Carica papaya*) latex as previously described [25]. N- α -tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) was from Fluka (Buchs, Switzerland). All the other chemicals were of analytical grade.

5.2. Methods

5.2.1. Phl p 1 preparation and purification

4.0 g of timothy grass (*Phleum pratense*) pollen were defatted with petroleum ether (~200 mL) using a soxhlet extractor and then dried under vacuum overnight. The protein extraction was carried out during 2 hours under moderate stirring at 25 °C in a 0.02 M Tris-HCl buffer (pH 8.0), containing 1 mM EDTA. The proportion of pollen to extraction buffer was 1:20 (w/v). After extraction, the sample was centrifuged (40,000 $\times$ g, 4 °C, 30 min). The insoluble pellet was discarded and the supernatant was freeze-dried (–60 °C) during 72 hours. The freeze-dried extract was resolubilized under stirring during 40 minutes at 25 °C in 100 mL of a 0.02 M Tris-HCl buffer at pH 8.0 containing 1 M ammonium sulfate. The solution was then filtered (0.45 μm). The filtrate constituted the crude extract.

5.2.2. Hydrophobic interaction chromatography on Phenyl Sepharose 6 fast flow

The crude extract (95 mL) was applied onto a (16 $\times$ 2.6 cm i.d.) Phenyl Sepharose 6 Fast Flow (high sub) column pre-equilibrated with a 0.02 M Tris-HCl buffer at pH 8.0 containing 1 M ammonium sulfate. The unbound material was washed away by the pre-equilibrating buffer and the elution of the bound proteins was carried out with water. The elution flow was 5 mL/min and fractions of 15 mL were collected. The chromatographic fractions were analyzed by measuring the absorbance at 280 nm.

5.2.3. Size-exclusion chromatography on Superdex 75

After hydrophobic interaction chromatography, the fraction corresponding to the unbound protein material and reported to contain Phl p 1 and Phl p 2/3 [59], was dialyzed against water (membrane cut off: 3.5 kDa) and concentrated by ultrafiltration (Amicon system, membrane cut off: 3.0 kDa). The concentrated solution was filtered through

a 0.45 μm diameter acrodisc, divided into three aliquots before to be fractionated by gel filtration chromatography on a (40 $\times$ 2.6 cm i.d.) Superdex 75 column pre-equilibrated with a degassed 50 mM ammonium hydrogen carbonate solution. Elution was carried out using the same equilibrating solution at a flow rate of 1.3 mL/min. Fractions of 4 mL were collected and their absorbances were measured at 280 nm. The pool containing Phl p 1, identified on the basis of its molecular weight, was rechromatographed on the same column under the same experimental conditions. The column was calibrated using bovine serum albumin (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa) and horse heart cytochrome c (12.4 kDa).

5.2.4. Affinity chromatography on STI-sepharose 4B

The STI-Sepharose 4B column was prepared in our laboratory by coupling STI to NHS-activated Sepharose 4B gel following the manufacturer's recommendations. Briefly, STI was dissolved in 0.1 M NaHCO_3 buffer at pH 8.3 and mixed with the activated gel which was pre-washed with 15 medium volumes of cold 1 mM HCl solution. The pH was adjusted to 8.3 and the coupling reaction was kept for 3 hours at room temperature under constant stirring. After the coupling was completed, the medium was kept in 100 mM Tris-HCl buffer pH 8.5 for 3 hours to block any non-reacted groups. The medium was washed by alternating 6 cycles of 3 $\times$ 1 medium volumes of 100 mM Tris-HCl buffer at pH 8.0 and 100 mM sodium acetate buffer at pH 5.0. Finally, the gel was packed in a column and further washed by alternating 100 mM Tris-HCl at pH 8.0 containing 0.5 M NaCl and a 3 mM HCl solution before to be equilibrated with 50 mM Tris-HCl buffer at pH 7.4.

The Phl p 1-containing pool obtained after rechromatography on Superdex 75 was dialyzed against the binding buffer (50 mM Tris-HCl pH 7.4) and applied onto the column of STI-Sepharose 4B column (7 $\times$ 1.2 cm i.d.) pre-equilibrated with eight volumes of the binding buffer. The column was first washed with the binding buffer and then with the binding buffer containing 500 mM NaCl. The bound material was eluted with a 3 mM HCl solution and collected in the presence of 100 mM Tris-HCl buffer at pH 8.0 to reach a final pH in between 7.0 and 8.0. During the binding and the elution steps, the flow rate was adjusted to 0.5 mL/min and fractions of 3 mL were collected and analyzed by absorbance at 280 nm.

5.2.5. SDS-PAGE experiments

The crude extracts and the different samples obtained after hydrophobic interaction, size-exclusion and affinity chromatographies were analyzed by electrophoresis. The experiments were performed using pre-cast gels (ExcelGel, 245 $\times$ 11 $\times$ 0.5 mm, gradient 8–18%) with the Multiphore II kit from GE Healthcare. The running conditions were 600 V, 50 mA and 35 W at a constant temperature of (15 $\pm$ 0.1) °C. Protein detection was performed according to the manufacturers' instructions using the silver staining kits from GE Healthcare or from Bio-Rad. The MS-compatible staining kit from Bio-Rad laboratories was used to perform mass spectrometry analyses. The molecular weight standards were hen egg white lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), bovine carbonic anhydrase (31.0 kDa), hen egg white ovalbumin (45.0 kDa), bovine serum albumin (66.2 kDa) and rabbit muscle phosphorylase b (97.4 kDa).

5.2.6. In-gel protein digestion and MALDI-TOF/MS analysis

The silver-stained protein spots of interest were cut out and 'in-gel' digested as previously described [26]. Peptides from digested proteins were solubilized in 2 μL of 0.5% trifluoroacetic acid (TFA). Peptide mass fingerprints (PMF) were obtained with a M@ldi™ mass spectrometer (Micromass, Manchester, UK) working in reflection mode with 15 kV of source voltage, 2.5 kV of pulse voltage and 2 kV of reflecting voltage. A saturated solution of α -cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.1% TFA was used for the matrix. A total of 1 μL

of the matrix and sample solution were mixed in a 1:1 (v/v) ratio and applied onto the 96 target wells. Mass accuracy for PMF analysis was 0.1 Da with external calibration, and internal calibration was carried out using enzyme autolysis peaks, resolution was 11000. The resulting peptide masses were automatically searched for, in a local copy of the Swiss-Prot, NCBI databases using the Protein Lynx global server and the Protein Probe (Micromass Ltd., Manchester, UK) and Mascot (<http://www.matrixscience.com>) search engines. The research was carried out in all species. One missed cleavage per peptide was allowed, a mass tolerance of 50 ppm was used and some variable modifications were taken into account such as carbamidomethylation of cysteines and oxidation of methionines. Protein identification results were manually evaluated. Only identification results with a confidence level above 95% were confirmed as positive hits.

5.2.7. LC-MS/MS analysis using an ion trap mass spectrometer

40 µg of sample were vacuum centrifuged to almost dryness, then resuspended in 6 M guanidine HCl/25 mM ammonium bicarbonate (AmBic), reduced with 2 mM TCEP, then alkylated with 5 mM iodoacetamide. After diluting to 1.5 M guanidine HCl/25 mM AmBic, 1 µg Trypsin (Promega) was added for overnight digestion. Digestion was stopped by addition of 5% formic acid.

The STI-Sepharose purified Phl p 1 sample solution (same sample deposited on SDS-PAGE) was treated, after carbamidomethylation, with trypsin (1/50) overnight at 37 °C. The tryptic peptides were analyzed by nanoLC-MS/MS using a LC Ultimate 3000 system (Dionex, United States) coupled to a HCT ultra plus mass spectrometer (Bruker Daltonics, Germany). 6 µL of each fraction were loaded onto a precolumn (C18 Trap, 300 µm ID × 5 mm, Dionex, United States) using the Ultimate 3000 system, delivering a flow rate of 20 µL/min of loading solvent. After a 10 min desalting step, the precolumn was switched online with the analytical column (75 µm ID × 15 cm PepMap C18, Dionex, United States) equilibrated in 96% of solvent A (0.1% (v/v) formic acid in HPLC-grade water) and 4% of solvent B (80% (v/v) acetonitrile, 0.1% (v/v) formic acid in HPLC-grade water). Peptides were eluted from the precolumn to the analytical column and then to the mass spectrometer with a gradient from 4% to 57% of solvent B for 50 min and 57% to 90% of solvent B for 10 min, at a flow rate of 0.3 µL/min delivered by the Ultimate pump.

The separated peptides were then analyzed on-line using an ion-trap mass spectrometer. This mass spectrometer was used in the positive mode and spectra were acquired in a data dependent manner: MS scan range = 300–1500 *m/z*, maximum accumulation time = 200 ms, ICC target = 200 000. The top 5 most intense ions in the MS scan were selected for MS/MS in dynamic exclusion mode: mode = ultrascan, absolute threshold = 75 000, relative threshold = 1%, excluded after spectrum count = 1, exclusion duration = 0.3 min, averaged spectra = 5, ICC target = 200,000. Peptide peaks were detected and deconvoluted automatically using the Data Analysis 3.4 software (Bruker). The mass list was generated automatically in the Mascot Generic Files format and searched against a local copy of the NCBI nr released 20080608 database using an in-house Mascot 2.2 server (Matrix Science) for protein identification (*p* < 0.05). The default search parameters used were: taxonomy = none, enzyme = trypsin, maximum missed cleavages = 1, fixed modification = Carbamidomethyl (C), variable modification = Oxidation (M), peptide mass tolerance ± 1.5 Da, fragment mass tolerance ± 0.5 Da, peptide charge = 1+, 2+ and 3+; instrument = ESI-TRAP. Only sequences identified with a Mascot score of at least 50 were considered. For each protein identified from one single peptide, MS/MS spectra were manually evaluated.

5.2.8. LC-MS/MS analysis using a new generation quadrupole time-of-flight (QqTOF) TripleTOF 5600 mass spectrometer

5 µL of sample (2 µg) was analyzed by an AB Sciex 5600 TripleTOF mass spectrometer interfaced with an Eksigent Nanolc system. Briefly,

after injection, peptide mixtures were transferred onto the analytical C18-nanocapillary HPLC column (C18 Acclaim PepMap100, 75 µm I.D. × 15 cm, 3 µm particle size, 100 Å pore size, Dionex, Sunnyvale, CA) and eluted at a flow rate of 300 nL/min using the following gradient: at 3% solvent B in A (from 0 to 13 min), 3–7% solvent B in A (from 13 to 16 min), 7–25% solvent B in A (from 16 to 48 min), 25–40% solvent B in A (from 48 to 65 min), 40–90% solvent B in A (from 65 to 75 min) and at 90% solvent B in A (from 75–85 min), with a total runtime of 120 min including mobile phase equilibration. Solvents were prepared as follows, mobile phase A: 2% acetonitrile/98% of 0.1% formic acid (v/v) in water, and mobile phase B: 98% acetonitrile/2% of 0.1% formic acid (v/v) in water. Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data was acquired using an ion spray voltage of 2.2 kV, curtain gas of 20 PSI. For data-dependent acquisition, survey scans were acquired in 250 ms and as many as 20 product ion scans were collected if exceeding a threshold of 150 counts per second and with a 2+ to 5+ charge-state. Total cycle time was fixed to 1.3 sec. Four time bins were summed for each scan at a pulser frequency value of 15.420 kHz through monitoring of the 40 GHz multichannel TDC detector with four-anode/channel detection. A sweeping collision energy setting of 35 ± 15 eV was applied to all precursor ions for collision-induced dissociation.

Mass spectrometric data was also analyzed using the database search engine ProteinPilot using the Paragon algorithm (4.0.0.0, 459). The following sample parameters were used: trypsin digestion, cysteine alkylation set to iodoacetamide, urea denaturation and acetylation emphasis. Trypsin specificity was assumed as C-terminal cleavage at lysine and arginine. Processing parameters were set to "Biological modification" and a thorough ID search effort was used. The peptide and fragment mass tolerances were < 10 ppm. During the search, Protein Pilot performs an automatic mass recalibration of the data sets based on highly confident peptide spectra. Specifically, a first search iteration is done to select high confidence peptide identifications that are used to recalibrate both the MS and MS/MS data, which is automatically re-searched. All data files were searched using the SwissProt 2011_02. For database searches, a cut-off peptide confidence value of 95 was chosen.

5.2.9. Enzymatic activity towards peptide substrates

The amidolytic activity was assayed fluorimetrically by following the fluorophore amidomethylcoumarin (AMC) released from Boc-Gln-Ala-Arg-7-amido-4-methylcoumarin and from Z-Phe-Arg-7-amido-4-methylcoumarin substrates. Phl p 1 (0.035 µM), was incubated at (37 ± 0.1) °C with Boc-Gln-Ala-Arg-AMC (2.5 µM) or Z-Phe-Arg-AMC (2.6 µM) in a 50 mM Tris/HCl buffer at pH 8.0 containing 3 mM EDTA and 0.01% Brij 35. Enzymatic activities were measured by monitoring the release of AMC, the fluorogenic leaving group. Fluorescence measurements were performed on a Shimadzu spectrofluorimeter, model RF-5301PC. The excitation was set at 360 nm and the emission measured at 440 nm using excitation and emission slit widths of 3 nm. Control assays without enzyme were used as blanks.

5.2.10. Inhibitory activity measurements

The protease inhibitors: 20 µM pepstatin A, 3 mM benzamidin, 3 mM iodoacetamide, 3 mM iodoacetic acid, 0.5 mM HgCl₂, 10 µM mM E-64, 1 mM Pefabloc SC, 0.1 mM TPCK, 0.1 mM TLCK, 1 mM PMSF, 5 mM EDTA, 0.25 mM STI and 0.25 mM PPI were assayed, for 30 min, for their inhibitory activity, both on Phl p 1 obtained after rechromatography on superdex 75 and on the protein fraction specifically retained on the STI-Sepharose column, prior to the addition of Boc-Gln-Ala-Arg-AMC. After an incubation period of 30 min at (37 ± 0.1) °C, the residual enzymatic activity was measured as described above.

5.2.11. pH optimum measurements

The pH effect on the enzymatic activity was examined in the pH range 4.0–9.0 using the following buffers, containing 3 mM EDTA and 0.01% Brij 35, at a final concentration of 30 mM: sodium acetate buffer for pH 4.0–5.0, MES buffer for pH 6.0, MOPS buffer for pH 6.6, Tris–HCl for pH 7.5–8.5 and Glycine–NaOH buffer for pH 9.0. The activity was measured at $(37 \pm 0.1)^\circ\text{C}$, as described above.

5.2.12. Temperature optimum measurements

The effect of temperature on the enzymatic activity was tested at different temperatures ranging from 25°C to 45°C in a 50 mM Tris/HCl buffer containing 3 mM EDTA and 0.01% Brij 35 at pH 8.0, as described above.

5.2.13. Protein determination

Protein concentrations were determined spectrophotometrically using $\epsilon_{280\text{ nm}} = 46798\text{ M}^{-1}\text{ cm}^{-1}$ for Phl p 1, $\epsilon_{280\text{ nm}} = 16740\text{ M}^{-1}\text{ cm}^{-1}$ for soybean trypsin inhibitor and $\epsilon_{280\text{ nm}} = 26270\text{ M}^{-1}\text{ cm}^{-1}$ for papaya protease inhibitor. These values were calculated using informations from the SwissProt Data Bank (MW, Trp and Tyr chromophores content and S–S bonds) and the known ϵ values of the various chromophores [64]. Absorbances were measured with a Varian UV-Visible Cary 50 spectrophotometer. Protein quantification was also alternatively performed by using a DC protein assay kit from Bio-Rad Laboratories according to the manufacturer's instructions with BSA as standard protein.

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