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Analytical and preparative native polyacrylamide gel electrophoresis: Investigation of the recombinant and natural major grass pollen allergen Phl p 2

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot are amongst the most popular methods for allergen characterization, such as comparison of recombinant allergens with their natural counterparts. Native PAGE was evaluated as a possible robust and simple method offering high-resolution capacity for characterization of the major grass pollen allergen Phl p 2. Analytical separation of recombinant Phl p 2 provided a superior quality control in terms of homogeneity and, after Western blotting, immunoglobulin E (IgE) reactivity. Separation of natural Phl p 2 identified two major isoforms which were shown to have different *N*-terminal sequences and IgE-binding properties. After isolation using preparative native PAGE in combination with electro dialysis, both isoforms were investigated by specific proteolysis and reversed-phase high-performance liquid chromatography (RP-HPLC). The results demonstrate differences in the primary structures and that the recombinant counterpart corresponds exactly to one isoform. Analytical and preparative native PAGE thus proved to be powerful tools for the investigation of allergen isoforms and quality control of recombinant counterparts.

Keywords: Immunoglobulin E-binding / Isoform / Native polyacrylamide gel electrophoresis / Phl p 2 / Recombinant allergen
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1 Introduction

Type-1 allergic reactions caused by grass pollen allergens are a worldwide health problem in industrialized countries. The identification, molecular cloning and characterization of allergenic components is an important step towards improving diagnosis and therapy [1, 2]. The most common methods for the investigation of allergens are electrophoretic separations under denaturing conditions, such as SDS- and 2-D PAGE. Subsequent blotting of the proteins onto membranes offers the possibility for immunological investigation with immunoglobulin E (IgE) and IgG antibodies from allergic subjects or mAbs. However, the resolution of SDS-PAGE is limited because of separation according to molecular size. Therefore, allergens with similar size might not be adequately separated by this technique, for example, group

2/3 and 6 allergens from grass pollen with approx. 12 kDa [3]. Another limitation concerns the isoforms of allergens, which differ mainly in charge but not in size, probably due to variations in amino acid composition [3–5]. In these cases, IEF or 2-D PAGE is performed, e.g., for group 2/3 [6] and 6 [4], which are relatively complex and time-consuming methods. In contrast to SDS-PAGE, it is difficult to make use of the resolution capacity of these techniques on a preparative scale in order to allow subsequent investigation of proteins using other biochemical or immunological methods [7].

The most important small-molecular-weight allergens of timothy grass pollen, Phl p 2 [8], Phl p 6 [9] and Phl p 7 [10], have been cloned successfully. The low-molecular-weight grass pollen allergen Phl p 2 has now been investigated in detail using native PAGE developed directly from SDS-PAGE. The technique has been found to be robust, simple and highly reproducible. Special attention was focused on the comparison of natural Phl p 2 isoforms with the recombinant counterpart. Therefore, a method making use of preparative native PAGE followed by electro dialysis was developed. Since the native structure of proteins is preserved, this method enables subsequent biochemical and especially immunological investigation.

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Abbreviations: AIEC, anion-exchange chromatography; EAST, enzyme allergosorbent test; IgE, immunoglobulin E; SEC, size-exclusion chromatography

2 Materials and methods

2.1 Expression and purification of recombinant allergens

The cDNA sequence of Phl p 2 [8] was expressed in *Escherichia coli* as an untagged molecule. Soluble rPhl p 2 containing supernatant of the bacterial lysate was adjusted to 1 M ammonium sulfate and applied to a phenyl Sepharose high-performance column (Amersham Biosciences, Freiburg, Germany). The flow through fraction contained rPhl p 2 and was directly applied to another hydrophobic interaction chromatography with Source 15 PHE (Amersham Biosciences). Elution of bound rPhl p 2 from the column was achieved with 20 mM Tris/HCl, pH 8.0. After desalting with Sephadex G-25 (Amersham Biosciences), protein-containing fractions were further purified by anion-exchange chromatography (AEX) with Source 15 Q (Amersham Biosciences). Elution was performed with a gradient ranging from 0 to 500 mM NaCl in phosphate buffer over 10 column volumes. Two fraction pools obtained after AEX were finally purified by size-exclusion chromatography (SEC) using Superdex 75 (Amersham Biosciences) eluted with 0.9% NaCl. Purified rPhl p 2 fractions were stored at -80°C until use. The Phl p 6 encoding cDNA [9] was cloned into a pProEx Ht expression vector (Gibco BRL, La Jolla, CA, USA). Expression and purification were conducted as described previously [11].

2.2 Isolation of natural Phl p 2/3/10

Grass pollen extracts were produced by Allergopharma (Reinbek, Germany). The purification of group 2/3 and 10 allergens was performed as described previously [6].

2.3 Native isoelectric focusing

Native analysis of proteins according to isoelectric points was performed using agarose-based gels containing 4% v/v Servalyt (Boehringer Ingelheim Bioproducts, Heidelberg, Germany) with a 1:2 mixture of pH gradients ranging from 4–7 and 4–9.

2.4 SDS-PAGE and Western blots

Proteins were separated by SDS-PAGE (size $120 \times 80 \times 0.8$ mm) with the buffer system of Laemmli [12], which is in accordance with the European Pharmacopoeia [13]. The stacking gel (5%T, 4%C) was comprised of 125 mM Tris/HCl, 0.1% w/v SDS, pH 6.8, the resolving gel (15%T, 4%C) of 340 mM Tris/HCl, 0.1% w/v SDS, pH 8.8 and the

electrophoresis buffer of 25 mM Tris, 192 mM glycine, 0.1% w/v SDS. The five times concentrated sample buffer contained 50% v/v glycerol, 0.3 M Tris/HCl, 10% w/v SDS, 0.05% w/v bromophenolblue, pH 6.8. Separation was conducted at 20 mA for approx. 90 min at room temperature. After electrophoresis, proteins were either stained using colloidal Coomassie (Roth, Karlsruhe, Germany) or subsequently transferred onto a 0.2 μm supported nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) by semidry blotting for 20 min at 2 mA/cm². Prior to blotting, the gel was equilibrated in 300 mM Tris, 20% v/v methanol and the membrane in 25 mM Tris, 20% v/v methanol. Blotting was conducted by using 3MM Whatman (Maidstone, UK) paper soaked with the same solutions and an anode buffer comprised of 40 mM aminohexanoic acid, 20% v/v methanol.

2.5 Native PAGE

Separation of proteins by PAGE (13.75%T, 4%C) under nondenaturing conditions was performed similar to SDS-PAGE, except that SDS and DTT were totally excluded. Electrophoresis was conducted at 10 mA at room temperature, resulting in a separation time of approx. 180 min. After addition of native sample buffer (5 $\times$), samples were directly applied to the gel without any preconditioning. Preparative native PAGE was conducted with a sample load of up to 0.2 mg protein/cm using a preparative comb. After electrophoretic separation, the outer lanes were cut and stained in order to locate the proteins. Protein-containing gel strips were subsequently placed into dialysis tubes (3.5 kDa cutoff), filled with 10 mL native PAGE running buffer and electroeluted at 5 mA/cm for 4 h in the same buffer using a submarine electrophoresis chamber (Biometra, Göttingen, Germany). In order to remove running buffer, dialysis bags were placed in distilled water for two consecutive periods of 2 h. Concentration of eluted proteins was conducted with ultrafiltration devices, Vivaspin 6 (5 kDa cut off; Vivascience, Göttingen, Germany).

2.6 Immunological assays

After transfer onto nitrocellulose or PVDF membranes, free binding sites were blocked by incubation in Tris-buffered saline (TBS) supplemented with 0.05% v/v Tween 20 (TBST). Identification of allergens was performed using a polyclonal rabbit antiserum specific for rPhl p 2 diluted 1:2000 in TBST. Incubation with 1:10 diluted individual sera obtained from grass pollen allergic subjects or patients' pooled serum was carried out overnight. After washing, bound antibodies were detected with alkaline

phosphatase-conjugated monoclonal mouse anti-human IgE (Allergopharma) diluted 1:2000, goat anti-mouse IgG/M (diluted 1:10 000) or goat anti-rabbit IgG (Dianova, Hamburg, Germany). The binding patterns were visualized using a substrate solution consisting of NBT/BCIP (Gibco-BRL, Karlsruhe, Germany) in 100 mM Tris/HCl-buffered saline solution (pH 9.5). Densitometric quantification of signals was conducted using a BioDocAnalyze system (Biometra, Göttingen, Germany). The relative IgE reactivities of allergens were evaluated with an enzyme allergosorbent test (EAST) inhibition assay using rPhl p 2/3 as a solid phase and a 1:2 diluted patient serum. The assay was performed as described elsewhere [14].

2.7 N-Terminal amino acid sequencing

Microsequencing of excised protein bands blotted onto a PVDF membrane (Bio Rad, München, Germany) was performed using a 473 A protein sequencer equipped with an on-line PTH amino acid analyzer (Applied Biosystems, Weiterstadt, Germany).

2.8 Specific proteolysis and RP-HPLC

Proteolysis of purified proteins was performed by addition of 0.025% w/w endoproteinase Lys-C or trypsin (Sigma, Taufkirchen, Germany) according the manufacturer's instructions. Separation was conducted on an HPLC (Kontron Instruments, Neufahrn, Germany) equipped with a CC125/3 Nucleosil 300-5 C8 column (Macherey-Nagel, Düren, Germany). The column was equilibrated with distilled water/0.1% TFA at 0.7 mL/min, loaded with 10 µg protein and eluted using a linear gradient over 10 column volumes with acetonitrile/0.7% TFA. Detection was carried out at 280 nm.

3 Results and discussion

3.1 Investigation of recombinant Phl p 2

The recombinant allergen Phl p 2 was produced in *E. coli* and subsequently purified. After two different hydrophobic interaction chromatography steps, AIEC served for intermediate purification (Fig. 1A). Two major peaks eluting with 18 mS/cm and 26 mS/cm both contained rPhl p 2 as judged by SDS-PAGE and Western blot (not shown). The pooled AIEC peak fractions were separately subjected to SEC (Fig. 1B). In both cases, the SEC chromatogram displayed a peak corresponding to the expected elution volume of Phl p 2 (Fig. 1B, peaks 1 and 3). The late eluting AIEC fraction contained an additional peak

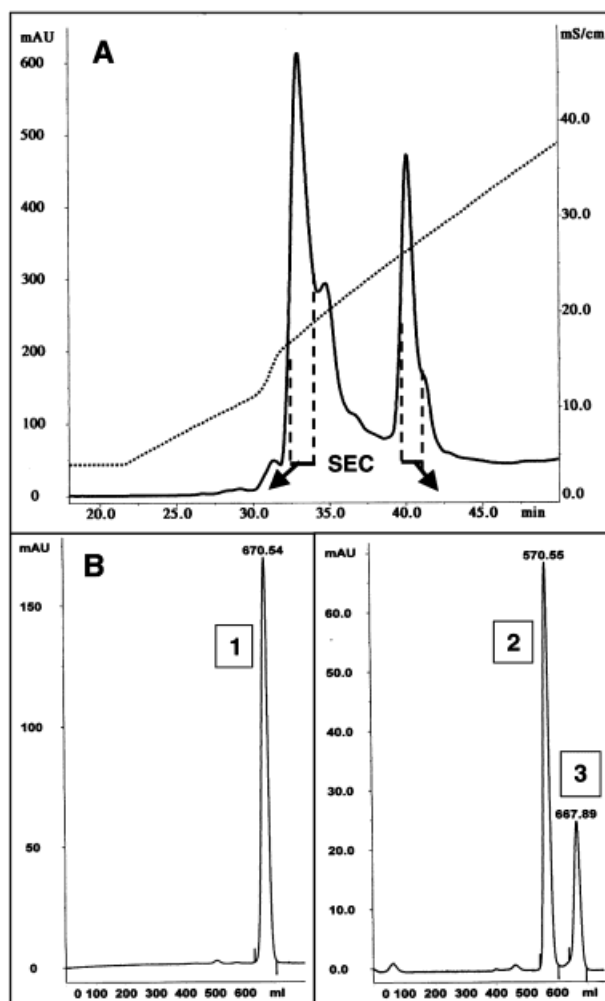


Figure 1. Purification of rPhl p 2. (A) AIEC of partially purified rPhl p 2. Bars and arrows indicate fraction pools subjected to SEC. (B) SEC chromatograms of AIEC fraction pools. Eluted peaks were designated as rPhl p 2 – 1, – 2, – 3.

with an estimated size of a Phl p 2 dimer (Fig. 1B, peak 2). Therefore, the recombinant preparation contained at least three biochemically distinguishable rPhl p 2 forms, although Phl p 2 contains no cysteine [8]. The same result has been obtained in four separate purifications thus substantiating the validity of this result.

In order to characterize the different rPhl p 2 fractions, SDS-PAGE with subsequent immunodetection (Fig. 2A) was compared with native PAGE separation (Fig. 2B). Neither Coomassie-stained SDS-PAGE analysis nor immunoblots showed differences between these fractions. In contrast, using native PAGE separation, fraction 2 appears to contain a monomer-dimer equilibrium of rPhl p 2 and fraction 3 tends to oligomerize, as indicated by the additional band detected using rPhl p 2-specific

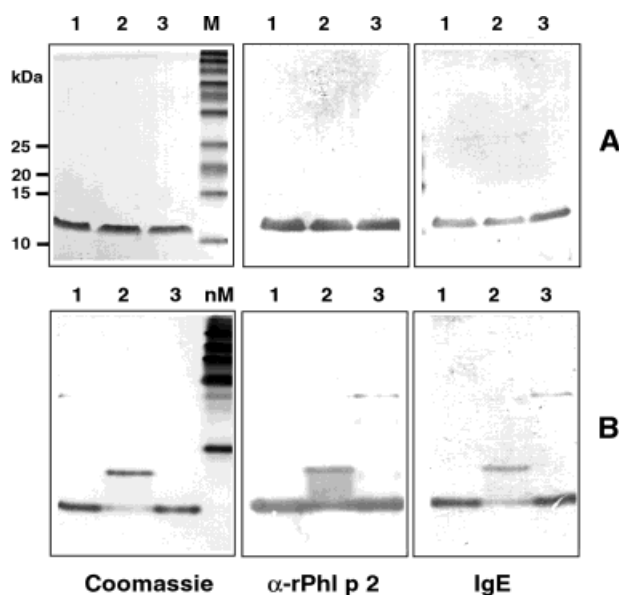


Figure 2. Characterization of rPhl p 2 fractions. (A) Fractions of rPhl p 2 obtained after AIEC and SEC (Fig. 1) were subjected to SDS-PAGE and either stained (left side), blotted and investigated with an rPhl p 2-specific anti-serum (center) or incubated with a serum obtained from a grass pollen allergic subject followed by IgE detection (right side). For staining 2 μ g and for blotting 1 μ g of each rPhl p 2 fraction was subjected to electrophoresis. (B) Native PAGE separation of rPhl p 2 fractions. Lane nM designates a native standard (IEF standard; BioRad).

antibody and IgE (Fig. 2B). In addition, it seemed that fraction 2 exhibited strongly decreased IgE reactivity. Therefore, an EAST inhibition assay was performed and compared with relative densitometric values determined from the IgE Western blots (Fig. 3, Table 1). Whereas fraction 1 and 3 were nearly identical in IgE-binding capacity, fraction 2 showed a strong reduction corresponding to the result obtained after native PAGE separation. The decrease of IgE reactivity in fraction 2 might be explained

Table 1. Determination of IgE-binding capacity of rPhl p 2 fractions by either EAST inhibition assay or densitometric analysis of Western blots obtained after SDS-PAGE and native PAGE

rPhl p 2 form (Fig. 1b)	μ g required for 50% IgE inhibition ^{a)}	Rel. dens. values SDS-PAGE (IgE) ^{b)}	Rel. dens. values nat. PAGE (IgE) ^{c)}
1	0.011	14.850	16.577
2	0.105	13.708	4.50
3	0.013	16.794	18.95

a) EAST-inhibition assay, solid-phase nPhl p 2/3

b) Fig. 2A

c) Fig. 2B

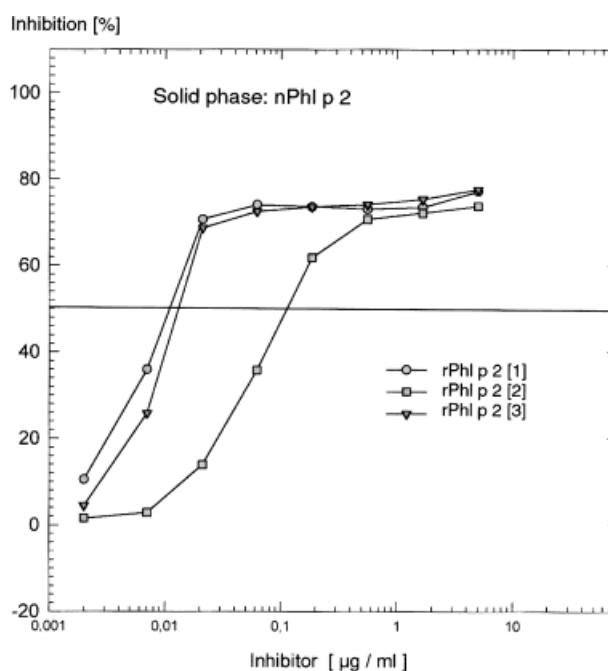


Figure 3. EAST inhibition assay of recombinant Phl p 2 fractions.

by either misfolding in terms of an altered structure compared to the natural molecule and/or masking of IgE epitopes due to dimerization. However, in addition to native PAGE separation structural differences were identified concerning surface charge as judged by AIEC elution profile and aggregation/misfolding potential as judged by SEC [15].

In summary, SDS-PAGE provided estimation of the purity with respect to host cell proteins, but not to product-related impurities. Native PAGE enabled clear differentiation of the rPhl p 2 fractions capable of dimerizing or even oligomerizing. In addition, IgE reactivity on the corresponding Western blot was more realistically reflected than in SDS-PAGE, which is probably due to the non-denaturing conditions and therefore conformation-preserving separation in contrast to SDS-PAGE [16]. This is especially important for allergen investigation, since IgE epitopes are known to depend mainly on the conformation [10].

3.2 Investigation of natural Phl p 2

In order to establish the suitability of native PAGE for comparison of recombinant Phl p 2 with natural Phl p 2 isoforms, purified proteins were separated by conventional SDS-PAGE and native PAGE (Fig. 4). Another recombinant low-molecular-weight grass pollen allergen, Phl p 6, was run in parallel for calibration purposes [11].

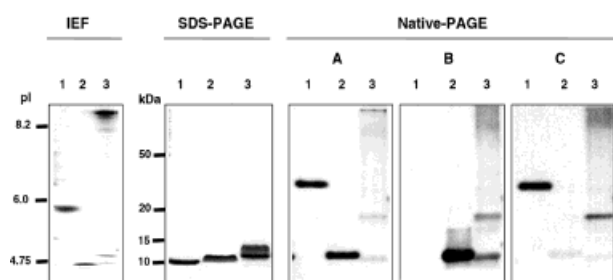


Figure 4. Separation of recombinant and natural allergens by IEF, SDS-PAGE and native PAGE. Native IEF and SDS-PAGE of rPhl p 6 (lane 1), rPhl p 2 (lane 2), nPhl p 2/3 (lane 3) followed by Coomassie staining. Native PAGE separation and (A) subsequent Coomassie staining; (B) incubation with an rPhl p 2-specific rabbit antiserum; and (C) IgE reactivity of a serum pool ($n = 5$) obtained from grass pollen allergic patients.

The separation by SDS-PAGE revealed only minor differences between purified allergens, since the molecular weights are located around 12 kDa [3]. Regarding native PAGE separation (Fig. 4A), recombinant allergens were clearly resolved, bearing in mind their very similar molecular masses. Natural group Phl p 2/3 was separated into two isoforms of group 2 (acidic pI), whereas the basic component Phl p 3 was not resolved or possibly did not migrate into the gel [3, 17, 18]. Another buffer system combined with reversed polarity may be employed to resolve Phl p 3 [14]. The recombinant Phl p 2 corresponded exactly with the fast migrating isoform, termed nPhl p 2-B (Fig. 4A). This was immunologically substantiated in that enhanced detection using an rPhl p 2-specific antiserum was observed for isoform B (Fig. 4B). Surprisingly, detection of group 2 IgE reactivity using a serum pool ($n = 5$) revealed that isoform B was clearly less IgE-reactive than the slow-migrating band, termed isoform A (Fig. 4C). Consequently, rPhl p 2 [8] might represent a molecule with advantages for therapeutic application, provided that T-cell reactivity is comparable to isoform B.

In order to substantiate the relatively low IgE reactivity of rPhl p 2-B, PVDF membrane strips carrying separated nPhl p 2 were incubated with individual sera (Fig. 5C). Again, nPhl p 2-A showed a more intensive reactivity than nPhl p 2-B, though equal amounts were present as judged by membrane staining (Fig. 5B) and Coomassie staining of the gel (Fig. 5D). Subsequent *N*-terminal sequencing of both Phl p 2 isoforms showed clear differences in both length and amino acid composition (Fig. 6). In addition, the sequences confirmed on the one hand the identity of Phl p 2 and on the other the observed correspondence of rPhl p 2 to the fast migrating natural isoform B.

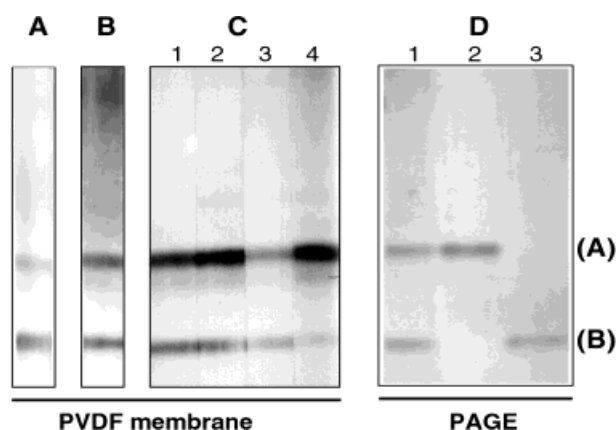


Figure 5. IgE-reactivity patterns of individual sera and preparative isolation of nPhl p 2 isoforms. A PVDF membrane carrying nPhl p 2 separated by native PAGE was (A) incubated with an rPhl p 2-specific rabbit antiserum; (B) stained with Auro dye; and (C) incubated with individual sera followed by IgE detection. (D) Coomassie-stained native PAGE with nPhl p 2/3 (lane 1), recovered Phl p 2-A (lane 2) and Phl p 2-B (lane 3).

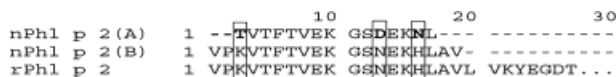


Figure 6. *N*-terminal sequencing of nPhl p 2 isoforms. After separation by native PAGE and subsequent blotting onto PVDF-membrane, the *N*-terminal sequences of nPhl p 2 isoforms were determined and compared with the deduced sequence of rPhl p 2 [8].

In order to investigate the isoforms chromatographically, a preparative native PAGE was conducted followed by electro dialysis (Fig. 5D). The approx. yield of each isoform was 40 μ g/gel, corresponding to a Phl p 2 recovery rate of 50%. Subsequent analysis of isolated natural isoforms and rPhl p 2 was performed using RP-HPLC. The samples were analyzed either directly (Fig. 7A) or after digestion using endoproteinase Lys-C (Fig. 7B) and trypsin (Fig. 7C). Detection was conducted by 280 nm in order to restrict detection to fragments containing amino acids with aromatic side chains. The retention times of both uncleaved and fragmented molecules substantiated the result obtained by native PAGE separation in that rPhl p 2 corresponds to nPhl p 2-B. Since it was only possible to detect internal fragments, as the *N*-terminal part lacks aromatic amino acids, differences between isoforms are clearly not limited to the *N*-terminal sequence.

In conclusion, native PAGE represents a highly suitable tool for the comparative investigation of the natural and recombinant forms of the major allergen Phl p 2. Moreover, the method enables both quality control and efficient separation of low recombinant molecular weight allergens Phl p 6 and Phl p 2.

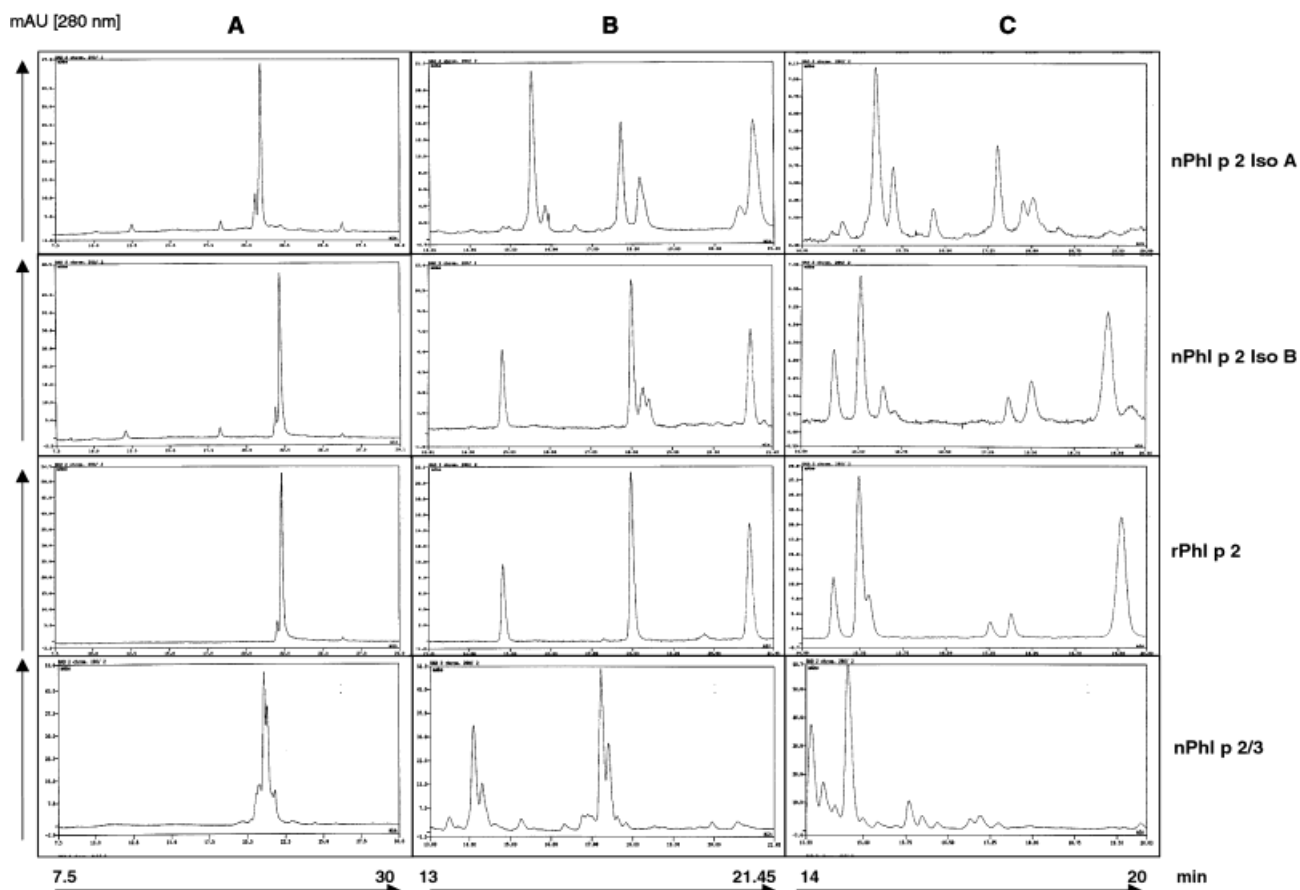


Figure 7. RP-HPLC analysis of recovered nPhl p 2 isoforms, rPhl p 2 and crude nPhl p 2/3/10 preparation. (A) Represent chromatograms of intact molecules; (B) after endoproteinase Lys-C-; and (C) after tryptic digestion as indicated.

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