Evaluation of the effects of phosphorylation of synthetic peptide substrates on their cleavage by caspase-3 and -7

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Caspases are a family of enzymes that play roles in cell death and inflammation. It has been suggested that in the execution phase of the apoptotic pathway, caspase-3, -6 and -7 are involved. The substrate specificities of two proteases (caspases 3 and 7) are highly similar, which complicates the design of compounds that selectively interact with a single enzyme exclusively. The recognition of residues other than Asp in the P1 position of the substrate by caspase-3/-7 has been reported, promoting interest in the effects of phosphorylation of amino acids in the direct vicinity of the scissile bond. To evaluate conflicting reports on this subject, we synthesized a series of known caspase-3 and -7 substrates and phosphorylated analogs, performed enzyme kinetic assays and mapped the peptide cleavage sites using internally quenched fluorescent peptide substrates. Caspases 3 and 7 will tolerate pSer at the P1 position but only poorly at the P2' position. Our investigation demonstrates the importance of peptide length and composition in interpreting sequence/activity relationships. Based on the results, we conclude that the relationship between caspase-3/-7 and their substrates containing phosphorylated amino acids might depend on the steric conditions and not be directly connected with ionic interactions. Thus, the precise effect of phospho-amino acid residues located in the vicinity of the cleaved bond on the regulation of the substrate specificity of caspases remains difficult to predict. Our observations allow to predict that natural phosphorylated proteins may be cleaved by caspases, but only when extended substrate binding site interactions are satisfied.

Introduction

Posttranslational modifications (PTMs) expand the possible functions of proteins after the translation process from RNA mainly by the covalent incorporation of chemical moieties (such as acylation, phosphorylation, and alkylation) or complex molecules (in the case of glycosylation and ubiquitylation) [1], resulting in changes in protein structures and properties. PTMs are involved in a wide ensemble of cellular processes including cell growth, differentiation, apoptosis, signal transduction, protein–protein and/or cell–cell interactions and communication between the intra- and extracellular environment [2–5]. Phosphorylation, which is one of the most widely studied types of posttranslational modifications, occurs in nearly one-third of proteins in eukaryotic cells at any given time [6]. Phosphorylation of t-serine and t-threonine is more widespread than that of t-tyrosine [7].

From a chemical perspective, phosphorylation results in a double negative charge located on the side chain of the modified residue (Figure 1), which indicates partial similarity to singly charged natural acidic amino acids (Asp/Glu). Moreover, comparative analysis of pro- and eukaryotic genomes has proven that some phosphorylation sites have evolved from acidic amino acids [8]. These findings
suggest that proteins interacting with Asp/Glu residues might also tolerate pSer/pThr/pTyr. Accordingly, it has been suggested that pSer and pThr may substitute for Asp and Glu residues that dictate specificity in proteolytic cleavage sites [8].

Several studies on the effect of phosphorylation on caspase-mediated cleavage have been performed. A proteomic approach applied to measure caspase cleavage observed in phosphorylated HeLa extracts dephosphorylated with λ bacteriophage phosphatase [9] revealed that the caspase-mediated cleavage of yes-associated protein 1 (YAP1) and Golgin-160 are negatively regulated by phosphorylation. In contrast, phosphorylation of mammalian Ste20-like protein kinase 3 (MST3) results in the improvement in cleavage efficiency by caspase-3 and -7. These enzymes were chosen because of their highly similar consensus motifs and crucial role in the majority of proteolytic events in apoptosis [10–12]. Nevertheless, further studies with synthetic peptides based on fragments of the MST3 sequence demonstrated that phosphorylation at the P2 and P4 positions had an inhibitory effect on caspase-mediated cleavage [9]. These findings suggest that the phosphorylation process might cause effects on caspase-mediated cleavage that are context specific and protein structure dependent.

Further analysis of the apoptotic phosphoproteome provided evidence that phosphorylation at the P3 position promotes proteolysis by caspase-8 [13].

The structure and sequence of protease substrates is influenced by the nature of the amino acids located adjacent to the scissile bond. Accordingly, alterations in this region can negatively or positively impact cleavage specificity and rates. Representative peptides derived from the caspase substrates poly(ADP-ribose) polymerase (PARP), growth arrest-specific protein-2 (Gas-2) and presenilin-2 (PS-2) substituted with phosphorylated L-serine residues in the P4–P1′ positions were used to examine the influence of the described modification on proteolytic efficiency [14]. Substitution of the Asp in the P1 position with pSer in the PARP-derived peptide sequence resulted in complete inhibition of cleavage by caspase-3, -7 and -8. A similar effect was observed for peptides containing unphosphorylated L-serine residues, which confirms the conservative preference of caspases for Asp located in the P1 position [15]. Replacement of Ser with pSer in the P1′ position of the PS-2 cleavage-site analog completely abolished the proteolysis mediated by caspases 3, 7 and 8. However, phosphorylated L-serine present at positions P3 of PS-2 and P4 of Gas-2-derived substrates was recognized by the mentioned caspases but poorly [14]. Other examples of substrate protection from caspase-mediated cleavage by the phosphorylation of amino acid residues adjacent to the scissile bond were also reported [16–24]. YAP1 and VIME, well-known caspase-3 substrates, predicted to contain phosphorylated residues close to the scissile bond, were analyzed to evaluate the binding free energies of caspase-3 and the appropriate ligand [25]. Replacement of Thr110 and Thr425 with pThr located in the P2 and P1′ positions of YAP1 cleavage-site peptides, respectively, resulted in a decrease in the binding free energy with caspase-3. Similarly, a more significant effect was observed when Ser87 was substituted with pSer at position P2′ of the VIME-derived peptide. These results suggest that phosphorylation in the P2, P1′ and P2′ positions may highly reduce caspase-mediated cleavage efficiency and, in particular, prevent cleavage entirely. One of the goals of our study was to test the hypothesis experimentally by synthesizing phosphorylated peptides.

In addition to their preferred cleavage after Asp residues, caspases are also able to cleave after Glu residues in both natural proteins and synthetic substrates, and even phosphoserine in a synthetic substrate [26]. We set out to examine these contradictory reports by examining the effects of phosphorylation at the specificity determining residues (P2–P2′ positions) of known caspase substrate sequences on changes in proteolytic cleavage by
caspase-3 and -7. We synthesized peptide sequences predicted as negatively regulated by phosphorylation at the P2, P1′ and P2′ positions in caspase-mediated cleavage [25] and experimentally examined previously published calculations. We also quantified the influence of the substitution of Asp in the P1 position with phosphorylated amino acids and phosphomimetics (Figure 2) in the context of an internally quenched fluorescent (IQF) peptide caspase substrate [27]. We expanded our studies on the influence of length and composition of synthetic peptide substrates modified at position P1.

Materials and methods

Reagents

All chemical reagents were obtained from commercial suppliers and used without further purification. Individual substrates were synthesized using as follows: Rink amide AM resin (100–200 mesh, 0.74 mmol/g), 2-chlorotrityl chloride resin (100–200 mesh, 1.6 mmol/g), piperidine (PIP, peptide grade), trifluoroacetic acid (TFA, peptide grade), and N,N-diisopropylethylamine (DIPEA, peptide grade) were bought from Iris Biotech GmbH (Germany); O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU, peptide grade) and O-(benzotriazole-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HBTU, peptide grade) were purchased from P3 BioSystems (USA); N-hydroxybenzotriazole (HOBt monohydrate, purity > 98%) was obtained from APEXBio (USA); 2,4,6-trimethylpyridine (collidine, peptide grade), trisopropylsilane (TIPS, purity 99%), N,N′-diisopropylcarbodiimide (DIC, peptide grade), and dimethyl sulfoxide (DMSO, purity ≥ 99.7%) were purchased from Sigma-Aldrich (Merck KGaA, Niemcy); and N,N′-dimethylformamide (DMF, peptide grade), dichloromethane (DCM, pure for analysis), methanol (MeOH, pure for analysis), acetonitrile (ACN, HPLC gradient grade), diethyl ether (Et2O, pure for analysis), acetic acid (AcOH, purity > 98%), and phosphorus pentoxide (P2O5, purity 98%) were bought from Avantor Performance Materials (Poland).

Figure 2. Chemical structures of phospho-\(\text{L-s}\)erine and phospho-\(\text{L-th}\)reonine analogs (Pma and Pmab, respectively) used in this study.

Fmoc-protected amino acids were purchased from various suppliers: Iris Biotech GmbH, Bachem (USA) and Creosalus (USA). For buffer solution preparation, 1,4-piperazinediethanesulfonic acid (PIPES, free acid), ethylenediaminetetraacetic acid dipotassium salt dihydrate (EDTA, purity ≥ 99%), and sodium chloride (purity ≥ 99.5%) from Sigma-Aldrich, sucrose from VWR International (Avantor, USA) and trisodium citrate dihydrate from Avantor were used.

Chemical synthesis

Designed fluorogenic substrates were synthesized using the solid-phase peptide synthesis (SPPS) method (on the Rink amide resin) according to the Fmoc-/tBu strategy. 7-Amino-4-carbamoylmethylcoumarin (ACC) was used as the fluorophore, and Fmoc-ACC-OH was synthesized as described previously [28]. Synthesis of tetrapeptide substrates was initiated by Fmoc removal using 20% piperidine/DMF, followed by the coupling of Fmoc-ACC-OH to the resin using HOBt and DIC in DMF. The 24-h reaction was carried out twice using 2.5 eq and 1.5 eq of reagents. After deprotection of the amino group of the fluorophore, the ACC resin was divided into five reaction vessels, where various amino acid derivatives located in the P1 position of the designed substrates were attached using HATU and colidine (2.5 eq) as coupling reagents (Fmoc-L-Ser(tBu)-OH, Fmoc-L-Ser(PO(OBzl)OH)-OH, Fmoc-L-Asp(OtBu)-OH, Fmoc-L-Glu(OtBu)-OH, and Fmoc-L-Pma(tBu)-2-OH). Peptide chains were further elongated in the coupling-deprotection cycles using HOBt and DIC (2 eq) in DMF, and the N-terminus of each substrate was acetylated using AcOH, HBTU and DIPEA in DMF (5 eq, 30 min).
The first step of the synthesis of IQF substrates was coupling of the Fmoc-amino acid (selected based on the peptide sequence) to the resin using HATU and collidine (2.5 eq) as coupling reagents. After the reaction was completed, peptide chains were elongated on the resin in a manner analogous to fluorogenic substrates (described above) with the exception of the acetylation step. To the N-terminal part of the substrate peptide chains were coupled the dipeptide Fmoc-ACC-βAla-OH synthesized previously, as follows: Fmoc-βAla-OH was attached to the 2-chlorotrityl chloride resin using DIPEA (2.5 eq) in anhydrous DCM in a 3-h reaction. The Fmoc-protecting group was removed using 20% piperidine in DMF, and Fmoc-ACC-OH was added to the resin with HATU and collidine (2.5 eq) in DMF. The final product was cleaved from resin using a standard cleavage mixture, precipitated in cold Et2O and lyophilized.

The 2-h condensation reaction between crude Fmoc-ACC-βAla-OH and peptidyl-resin was performed using HATU and collidine (2.5 eq) in DMF, and the final deprotection of amino groups was carried out using 20% piperidine/DMF. Ninhydrin tests were performed during the entire chemical synthesis to ensure that each reaction was completed. All synthesized substrates were cleaved from the resin using a TFA/TIPS/H2O cleavage mixture (95% : 2.5% : 2.5%, v/v/v), precipitated in cold Et2O, centrifuged, dried and lyophilized. After identification and purification, individual compounds were dissolved in DMSO at a concentration of 20 mM and stored at −20°C until use.

**Recombinant caspase expression and characterization**

Human caspases 3, 6, 7 and 8 were expressed and purified as previously described [29]. All caspases were active site-titrated using the broad spectrum inhibitor zVAD-fmk, following the protocol previously described [29]. Caspase assay buffer composition: 20 mM PIPES, 10 mM NaCl, 1 mM EDTA, 10% w/v sucrose, and 10 mM DTT (pH 7.2). The caspase-8 assay buffer was supplemented with 1 M sodium citrate to force dimerization and maintain high enzyme activity.

**MS and HPLC analysis**

Individual substrates were purified using a preparative HPLC system (Waters 1525 solvent delivery module and Waters 2489 detector; Waters, USA) equipped with a C8 column (300 Å, 10 μm, 250 mm × 21.2 mm; Waters). A linear gradient of 5–95% solution B in A over 30 or 40 min with a flow rate of 15 ml/min was used (solvent A — water/0.1% TFA, B — ACN/0.1% TFA). The purity of the compounds was confirmed using an LCMS Acquity QDa system (Waters e2695 solvent delivery module and Waters 2489 detector) equipped with a C4 column (300 Å, 5 μm, 250 mm × 4.6 mm; Waters) in a linear gradient of 5–95% solution B in A over 20 min, with a 1 ml/min flow rate. The substrates were dissolved in dimethyl sulfoxide.

The molecular weight of each substrate was confirmed by mass spectrometry; for this purpose, a Waters LCT premier XE ESI TOF high-resolution mass spectrometer was used.

**Substrate screening**

All the kinetic studies were performed using an fMax spectrofluorometer (Molecular Devices SpectraMax Gemini XPS) at 37°C and 96-well plates (Corning®) or 384-well plates (Corning®). The ACC fluorescence was monitored using 355 nm and 460 nm wavelengths (excitation and emission, respectively). Before each measurement, enzymes were preincubated in the assay buffer: for caspase-3, -6 and -7 (10% w/v sucrose, 20 mM PIPES, 100 mM NaCl, 1 mM EDTA and 10 mM DTT at pH 7.2–7.4) [30] for 10 min at 37°C and for caspase-8 for 20 min at 37°C (the buffer for caspase-8 was supplemented with 1 M sodium citrate) [31]. The individual substrate concentrations in each well were 1 μM and 10 μM, and the enzyme concentrations varied from 0.4 to 600 nM. Measurements were conducted for 30 min, and the linear part of each progress curve was used to determine the substrate hydrolysis rate. Kinetic assays were repeated two or three times, and the results are presented as the mean values with standard deviations.

**Determination of kinetic parameters (k_{cat}, K_M, k_{cat}/K_M) for individual substrates**

The kinetic parameters (k_{cat}, K_M, k_{cat}/K_M) of selected caspase-3 and -7 substrates were determined by measuring the fluorescence increase over time. Preincubation of enzyme in the appropriate buffer was followed by addition to the wells containing eight different substrate concentrations diluted at 2/3. The final enzyme concentration in the assay solution ranged from 1 to 500 nM, the starting concentration of substrates varied.
from 25 to 150 μM, and the total reaction volume was 100 μl. Substrate hydrolysis was measured for 30 min, and the linear part of each progress curve was used to calculate the kinetic parameters with the Michaelis–Menten equation using nonlinear regression analysis. Kinetic parameters were calculated using GraphPad Prism software. All the experiments were performed three times, and the average values with the SD are presented.

### Identification of the cleavage sites

For the experimental determination of the studied substrate cleavage sites, three samples each containing 150 μM appropriate peptide (S5_VIME, S6_[pS]VIME or (P1)_pSer; Tables 1 and 2) and caspase-3 at final concentrations of 30 nM, 400 nM and 1 μM, respectively, were prepared. Caspase-3 was previously activated in buffer (10% w/v sucrose, 20 mM PIPES, 100 mM NaCl, 1 mM EDTA and 10 mM DTT; pH 7.3) for 10 min at 37°C. After two (S5_VIME substrate), two and a half (S6_[pS]VIME) and 5 h of incubation ((P1)_pSer), samples were diluted with 500 μl of distilled water and analyzed via an LCMS Acquity QDa system (Waters, USA) equipped with a C4 column (300 Å, 5 μm, 250 mm × 4.6 mm; Waters). The 5-min isocratic elution was followed by a linear gradient of solution B in A from 5% to 95% (A — water/0.1% HCOOH, B — ACN/0.1% HCOOH) over 20 min, with a flow rate of 1 ml/min.

Analogous experiments were performed for the (P1)_pSer peptide and caspase-7 at concentrations of 140 μM and 1 μM, respectively, and after 5 h of incubation.

### Table 1. Data summary of kinetic parameters determined in the current study and total binding free energies of YAP1- and VIME-derived substrates determined for caspase-3

| Symbol and sequence of substrate | Cleavage site in natural substrate | Phosphorylation site | Caspase-3 | Caspase-7 |
|---------------------------------|-----------------------------------|---------------------|-----------|-----------|
|                                 |                                   |                     | KM (μM)  | kcat/KM (M⁻¹ s⁻¹) | ΔGbinding [25] (kcal/mol) |
| S1_YAP1 ACCpβAVEDMDT^25GDTIK(Dnp)-NH2 | Asp^254-Thr^425 (YAP1) | —                   | 15.5 (± 1.25) | 110 000 (± 4770) | −41.47 |
| S2_[pT]YAP1 ACCpβAVEDMTP^25GDTIK(Dnp)-NH2 | Asp^254-Thr^425 (YAP1) | P1’                 | 17.6 (± 0.64) | 205 (± 2) | −4.14 |
| S3_YAP1 ACCpβAQAAS^110DAGTAGK(Dnp)-NH2 | Asp^111-Ala^112 (YAP1) | —                   | 16.2 (± 0.69) | 2280 (± 54) | −42.78 |
| S4_[pT]YAP1 ACCpβAQAASp^110TAGTAGK(Dnp)-NH2 | Asp^111-Ala^112 (YAP1) | P2                 | NH⁺ | NH⁺ | −25.16 |
| S5_VIME ACCpβAQDSVDF^87NHDK(Dnp)-NH2 | Asp^85–Phe^86 (VIME) | —                   | 13.3 (± 0.52) | 131 000 (± 3140) | −40.33 |
| S6_[pS]VIME ACCpβAQDSVDFp^87NHDK(Dnp)-NH2 | Asp^85–Phe^86 (VIME) | P2’                | 16.4 (± 1.30) | 2240 (± 150) | 2.36 |

*Substrate was not hydrolyzed even though a high concentration of enzyme was used in this study.

### Table 2. Kinetic parameters determined for the caspase-3 and -7 IQF peptide substrates modified in the P1 position

| Peptide | Caspase-3 | Caspase-7 |
|---------|-----------|-----------|
|         | KM (μM)  | kcat/KM (M⁻¹ s⁻¹) | KM (μM)  | kcat/KM (M⁻¹ s⁻¹) |
| (P1)_Asp ACCpβADEVDGVK(Dnp)-D-NH₂ | 6.01 (± 0.29) | 987 000 (± 74 200) | 6.05 (± 0.40) | 86 500 (± 4060) |
| (P1)_Glu ACCpβADEVEGVK(Dnp)-D-NH₂ | 12.8 (± 1.27) | 23 400 (± 2000) | 15.7 (± 1.46) | 13 400 (± 1120) |
| (P1)_pSer ACCpβADEVPSGVK(Dnp)-D-NH₂ | 8.76 (± 0.09) | 225 (± 13) | 12.2 (± 0.28) | 99 (± 6) |
Results

Phosphorylation of Thr\textsuperscript{110} and Thr\textsuperscript{425} of YAP1-derived peptides as well as Ser\textsuperscript{87} of VIME-derived compound results in rapid decrease in proteolysis by caspase-3

YAP1 was reported as a caspase-3 substrate with cleavage sites QASTD\textsuperscript{111}↓AGTAG [32] and VDEMD\textsuperscript{424}↓TGDTI [9] recognized by caspase-7 and initiator caspase-8 as well, but in a significantly less efficient manner. The second of the caspase-3/-7-like substrates selected in this study, the VIME protein, is hydrolyzed within the motif QDSVD\textsuperscript{85}↓FSNHD [33]. Based on previous findings describing the negative effect of phosphorylation in the vicinity of the scissile bond, we decided to investigate the influence of L-threonine phosphorylation at positions P2 and P1\textsuperscript{0} of YAP1-derived peptides and L-serine in the P2\textsuperscript{0} position of the VIME analog on caspase-3 cleavage efficiency. Therefore, we synthesized six IQF peptide substrates, with three of them containing phospho-amino acids embedded within previously reported caspase-selective sequences [25] (Table 1). We observed that the compound S\textsubscript{4}_[pT]YAP1 with a phospho-L-threonine residue located in the P2 position was not recognized by caspase-3 (Figure 3). This enzyme did not hydrolyze the peptide bond after the Asp\textsuperscript{111} residue, which led us to conclude that the described modification resulted in pronounced inhibition of caspase-mediated cleavage. A rapid decrease in cleavage by caspase-3 was also observed for the S\textsubscript{2}_[pT]YAP1 compound phosphorylated at position P1\textsuperscript{0}, which exhibits an over 530-fold lower \(k_{cat}/K_M\) value than the unphosphorylated analog (Table 1). Interestingly, in the case of the S\textsubscript{6}_[pS]VIME peptide, we demonstrated a \(K_M\) value of 16.4 ± 1.30 \(\mu\)M, which is highly comparable to that for the unmodified VIME-derived compound (13.3 ± 0.52 \(\mu\)M; Table 1) and suggests that caspase-3 has a similar affinity to both substrates. The replacement of Ser with pSer in the P2\textsuperscript{0} position of the VIME-derived peptide resulted in a 58-fold decrease in cleavage by caspase-3 (\(k_{cat}/K_M\), Table 1). Moreover, we performed screening using caspase-6, -7 and -8 and found that these enzymes did not hydrolyze any of the newly synthesized compounds (Figure 3), which suggests that substrate S\textsubscript{6}_[pS]VIME might be slightly but selectively recognized by caspase-3. Loss of catalytic efficiency of cleavage between P1 Asp > Glu > pSer is primarily a \(k_{cat}\) effect. Because pSer binds well but is cleaved inefficiently, we speculate that the phosphorylation of serine may cause substrates to act as competitive inhibitors in a physiological context. Naturally this speculation will need to be tested by appropriate future experiments.

Caspase-3 hydrolyzes the substrate designed based on the fragment of the VIME protein containing pSer in the P2\textsuperscript{0} position

For precise identification of the hydrolyzed peptide bond in the VIME-derived substrate with the sequence ACC-βAQDSVDFpS\textsuperscript{85}NHDK(Dnp)-NH\textsubscript{2} (peptide S\textsubscript{6}_[pS]VIME), we used a mass spectrometry-based
method. The tested compound with a calculated molecular mass of 1808.62 Da after two and a half hours of incubation with caspase-3 was divided into two major products at m/z values of 835.17 and 496.63 corresponding to the singly charged ion of ACC-βAQDSVD and doubly charged ion of FpS87NHDK(Dnp)-NH2 (Figure 4 and Supplementary Figure S1). We identified the cleavage site of the newly synthesized substrate S6$_{pS}$VIME, ACC-βAQDSVD↓FpS87NHDK(Dnp)-NH2, and simultaneously demonstrated that caspase-3 is able to hydrolyze peptides containing phospho-threonine in the P2$^0$ position, which, according to our current knowledge, has not yet been reported. As a control sample, we used an unphosphorylated substrate with an analogous sequence (S5_VIME) and confirmed the cleavage site after Asp$^{85}$ (ACC-βAQDSVD↓F87NHDK(Dnp)-NH2; Supplementary Figure S2). Notably, the concentrations of caspase-3 used in both assays and time of incubation with substrates largely varied, which is reflected by the determined cleavage efficiency values of each of the studied compounds (Table 1).
Substitution of acidic amino acid residues with phospho-L-serine at position P1 of internally quenched fluorescent substrates maintains caspase-3- and -7-mediated cleavage

To evaluate the influence of P1 phosphorylation on the efficiency of caspase-specific cleavage, we decided to synthesize a series of IQF peptide substrates based on the previously reported sequence ACC-BADEVD↓GVK (Dnp)D-NH₂ [27] containing the optimal recognition motif [34] as the fragment of the PARP protein pattern cleaved by caspase-3 and -7 (GDEVD↓GVDEV). Compounds with incorporated Asp and Glu in the P1 position were used as the control substrates. Synthesized peptides containing Ser, pSer, Thr, pThr, Pma and Pmab were applied to investigate the effects of P1 phosphorylation. We decided to include phospho-L-threonine and its mimetic (Pmab) to expand our studies but omitted the pTyr residue because of the more probable steric hindrance in the interaction with the active site of the enzyme, which was speculated previously [26]. Notably, substrates with Asp and Glu present in the P1 position were rapidly hydrolyzed by caspase-3 and less efficiently recognized by caspase-7. The replacement of acidic residues with Ser and Thr completely abolished cleavage mediated by the studied enzymes (Figure 5). A similar effect was observed for substrates containing pThr and Pmab derivatives, which confirms suggestions that phosphorylated l-threonine and its analog are probably too large to allow suitable conformations of the peptide chain to efficiently interact with both caspases. The most noteworthy influence of P1 phosphorylation on the retention of caspase-3-mediated cleavage was registered for modification with pSer (Figure 5). It should be emphasized that caspase-7 recognized phosphorylated l-serine in the P1 position as well but exhibited 2.3-fold lower catalytic efficiency, which was proven by the determined \( k_{cat}/K_M \) values (99 ± 6 M⁻¹ s⁻¹ and 225 ± 13 M⁻¹ s⁻¹ for caspase-7 and -3, respectively; Table 2). The \( K_M \) value determined for caspase-3 was almost 1.4-fold lower in comparison with that for caspase-7 (Table 2), which suggests analogous affinity of both mentioned proteases to interact with the substrate. Our obtained results might lead to the conclusion that peptide (P1)ₚSer is cleaved by both caspases 3 and 7, which has not been previously reported.

Both caspases 3 and 7 cleave the peptide bond located directly after a phospho-L-serine residue

To experimentally confirm the predicted cleavage site of the (P1)ₚSer substrate, we incubated it for 5 h with caspase-3 and -7 separately. After this time, we observed three signals on the mass spectrum (Figure 6) at \( m/z \) 801.09, 584.07 and 683.65 in the sample with caspase-3. The first one corresponds to the singly charged ion of...
ACC-\(\beta\)ADEV\(p\)S, and the second proves the presence of the GVK(Dnp)D-NH\(_2\) peptide fragment. The third signal comes from the doubly charged ion of the nonhydrolyzed substrate remaining in the sample. Its presence, in conjunction with the high enzyme concentration and elongated incubation time used in this study,

**Figure 6.** Chemical structure of the \(\text{P1}_{\text{p}}\text{Ser}\) substrate (A) and chromatograms illustrating the identification of cleavage sites hydrolyzed by caspase-3 (black) and -7 (pink) (B).

UV absorbance was measured at a wavelength of 254 nm. Signals at \(m/z\) 801.09 and 801.16 represent singly charged ions of ACC-\(\text{pADEVpS}\), whereas \(m/z\) 584.07 and 584.06 correspond to singly charged ions of the GVK(Dnp)D-NH\(_2\) compound. After five-hour incubations, the nonhydrolyzed ACC-\(\text{pADEVpS}\)GVK(Dnp)D-NH\(_2\) peptide was still present in the sample, which was proven by its doubly charged ions at \(m/z\) 683.65 and 683.67. Samples were prepared and analyzed as described in detail in the materials and methods section.
is in agreement with the poor kinetic parameters measured for this compound (Table 2). Similar effects were observed for the same peptide incubated with caspase-7 (Figure 6). We mapped the cleavage site ACC-βADEVPSP̄SV̄AK(Dnp)-D-NH₂ and therefore demonstrated that caspases 3 and 7 hydrolyze the bond located directly after the pSer residue in the sequence of IQF peptide substrates (Supplementary Figure S3). The obtained results are important in the identification of the influence of slight modifications within the recognition pattern required by tested proteases and provide experimental evidence for caspase-3- and -7-catalyzed hydrolysis with phospho-L-serine as the P1 amino acid present in the immediate vicinity of the scissile bond.

**Phosphorylation at position P1 of tetrapeptide substrates completely abolishes proteolysis by caspases 3 and 7**

We wondered whether caspase-3 and/or -7 are able to recognize P1 phosphorylated residues (pSer/Pma) in tetrapeptide substrates exhibiting shorter sequences, in comparison with the tested IQF substrates derived from the PARP cleavage pattern. We synthesized analogs of the well-studied caspase-3 and -7 substrate Ac-DEVD-ACC-NH₂ substituted in the P1 position with Glu, pSer, Ser and Pma. The peptides containing natural acidic amino acid residues (Asp, Glu), as well as L-serine, were considered control substrates. We performed enzyme kinetic assays to verify the cleavage efficiency of the synthesized peptides by caspase-3 and -7. As expected, the compounds with conserved P1 aspartic and glutamic acid residues were properly recognized and rapidly cleaved by both caspases. However, we noticed no proteolysis of other synthesized substrates by either caspase-3 or -7 (Supplementary Figure S4), even though high concentrations of substrates and enzymes were used in these assays. The obtained results are in opposition to the effect observed in the case of pSer present in the P1 position of the IQF substrate ((P1)_pSer), which is probably caused by shorter peptide chain and hence weaker interactions with the active site of caspases.

**Discussion**

The application of IQF substrates is a common approach used in the examination of the specificity profile of a wide range of proteases, including caspases [34–41]. Posttranslationally modified amino acids, as well as unnatural derivatives, expand this field of research because of their diverse chemical structures and the resulting broad spectrum of properties, which might help to identify slight differences in the substrate specificity of proteases exhibiting similar binding preferences [42].

Contradictory results proposed that phosphorylation of Ser in P1 is either promisive [26] or restrictive [14] of substrate cleavage by caspases. Our investigation demonstrates the importance of peptide length and composition in interpreting sequence/activity relationships (Table 3). Caspase-3 and -7 are able to cleave the long peptide (ACC-βADEVPSP̄SV̄AK(Dnp)-D-NH₂) but not the short peptide (Ac-DEV̄p̄S-ACC-NH₂). Possibly interactions between the caspase and the prime side of the substrates overcome a poor tolerance for pSer in P1. The poor recognition of short peptides is overcome by increasing peptide length. Indeed, this has been observed previously in the recognition of peptide substrate by Drosophila initiator caspase DRONC [43].

However, the results obtained in the current study demonstrate that both caspase-3 and -7 hydrolyze peptides containing phospho-L-serine residues, which has not yet been reported. Analysis of the determined $K_M$ values suggests that the binding of the (P1)_pSer peptide by caspase-3 is slightly stronger (1.4-fold) than that

| Peptide                        | Caspase-3 | Caspase-7 | Reference |
|-------------------------------|-----------|-----------|-----------|
|                               | $k_{cat}$ (s$^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$) | $k_{cat}$ (s$^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$) |          |
| GDEVp̄SḠDEV                   | Not hydrolyzed* | — | — | — | — | Not hydrolyzed† | [14] |
| biotin-WDEVp̄SḠVĒK(Dnp)      | 0.017 | 9.95 | 1700 | — | — | Not hydrolyzed | [26] |
| ACC-μADEVP̄SḠV̄K(Dnp)-D-NH₂    | 0.002 (± 0.0002) | 8.76 (± 0.09) | 225 (± 13) | 0.001 (± 0.0001) | 12.2 (± 0.28) | 99 (± 6) | Current study |

*In conditions 0.2 mM substrate and 50 nM enzyme after 16 h of incubation;
†Not hydrolyzed when 0.2 mM substrate was incubated with 1 μM caspase-7 for 16 h.
by caspase-7 and that the catalytic step is 2-fold faster, which is indicated by the $k_{cat}$ values (Table 3). These findings underline the subtle differences in the overlapping substrate specificity of the executioner caspases 3 and 7. These are likely do to their highly similar sequences, activation mechanisms and structures of the mature forms of the enzymes [44–46]. Caspases 3 and 7 cleave the peptide chain of the substrate directly after the pSer residue in ACC-[βADEVPs]GVK(Dnp)D-NH$_2$ (Figure 6). Interestingly, this effect was not observed when pSer was substituted with Ser, or Pma — its mimetic containing a methylene group instead of the oxygen atom originating from the l-serine side chain (Figure 5). Moreover, substrates modified in the P1 position with pThr or Pmab residues were not recognized by any of the studied enzymes. The S1 pocket of caspases is highly basic due to the side chains of Arg$_{179}$ and Arg$_{341}$ residues that are involved in its construction [44], which explains the preference for the l-aspartic acid residue located in the P1 position of the substrate. Similar interactions resulting in the formation of a salt bridge might occur with the side chain of phosphorylated amino acid residues present in the P1 position containing a negatively charged phosphate group. The described effects should be analogous for substrates with pSer as well as Pma, pThr and Pmab residues located at position P1. The results obtained in this study do not confirm this hypothesis; therefore, we speculate that the phospho-l-serine residue is not recognized by caspase-3 and -7 directly but might ensure sterically proper arrangement of the substrate peptide chain in the substrate pocket of the enzyme, or because the van der Waals radius of the phosphate group is larger than the radius of the carboxylated side chain of Asp.

The presented results suggest that the application of posttranslationally modified amino acids in the design of peptide substrates may alter their cleavability by caspases 3 and 7. The interactions between caspase-3/-7 and substrates containing phosphorylated amino acid residues appear to be difficult to investigate in detail. Therefore, the precise effect of phosphorylated amino acid residues placed in the P1 position of the substrate on the regulation of their specificity toward caspases remains difficult to predict. Although the phosphorylated substrate S6_[pS]VIME is cleaved ~58 times less efficiently than the unphosphorylated version, it is cleaved with similar efficiency to the unphosphorylated S3_YAP1 substrate. This indicates that phosphorylation could be used to fine tune cleavage efficiencies and specificity.

Conclusions

We confirmed that the replacement of natural acidic amino acids with phospho-l-serine in the P1 position of the IQF-type substrate designed based on the PARP sequence fragment can result in a compound hydrolyzed by caspase-3. Furthermore, we demonstrated that caspase-7 is able to cleave this substrate as well, which remains in agreement with the overlapping substrate specificities of the two most similar executioner caspases 3 and 7 but, on the other hand, is contrary to previously published reports (Table 3). Nevertheless, a tetrapeptide substrate containing pSer in the P1 position is recognized by neither caspase-3 nor -7, which implies the requirement for longer and more flexible substrate peptide chains to properly interact with the active site of caspases. Considering the presence of phosphorylated amino acid residues in the peptide chain of the caspase-3 substrate located farther from the scissile bond, we demonstrated that pSer at position P2’ causes a rapid decrease in proteolysis, but we simultaneously showed that this modification might maintain the cleavage mediated by the studied enzyme. The present findings suggest that phosphorylation should not be considered as modification causing complete inhibition of hydrolysis by caspases. Our observations allow us to predict that natural phosphorylated proteins may be cleaved by caspases, but only when extended substrate binding site interactions are satisfied.

Data Availability

The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary materials. Raw data were generated at the Wroclaw University of Science and Technology and are available from the corresponding author (MD) on request.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution
Marcin Drag: Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, investigation, methodology, writing — review and editing. Izabela Maluch: Data curation, formal analysis, validation, investigation, visualization, methodology, writing — original draft. Justyna Grzymska: Data curation, formal analysis, validation, investigation, writing — review and editing. Scott Snipas: Resources, writing — review and editing. Guy S. Salvesen: Resources, writing — review and editing.

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Abbreviations
IQF substrate, internally quenched fluorescent substrate; PARP, poly(ADP-ribose) polymerase; Pma, (S)-2-amino-4-phosphorybutanoic acid; Pmab, (2S,3R)-2-amino-3-methyl-4-phosphorybutanoic acid; VIME, vimentin; YAP1, yes-associated protein 1.

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