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Biomedicine & Diseases: Review

Prolyl endopeptidases

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Abstract. This review describes the structure and function of prolyl endopeptidase (PEP) enzymes and how they are being evaluated as drug targets and therapeutic agents. The most well studied PEP family has a two-domain structure whose unique seven-blade β-propeller domain works with the catalytic domain to hydrolyze the peptide bond on the carboxyl side of internal proline residues of an oligopeptide substrate. Structural and functional studies on this protease family have elucidated the mecha- nism for peptide entry between the two domains. Other structurally unrelated PEPs have been identified, but have not been studied in detail. Human PEP has been evalu- ated as a pharmacological target for neurological diseases due to its high brain concentration and ability to cleave neuropeptides *in vitro*. Recently, microbial PEPs have been studied as potential therapeutics for celiac sprue, an inflammatory disease of the small intestine triggered by proline-rich gluten.

Keywords. Prolyl endopeptidase, β-propeller, neurological disorder, celiac sprue, gluten.

Introduction

The cyclic amino acid proline plays a critical physiologi- cal role by protecting peptides from proteolytic degrada- tion. Thus, proline is present in biologically active pep- tides for a wide range of diseases, including depression, Parkinson's disease, and celiac sprue [1–3]. The prolyl endopeptidase (PEP, also known as prolyl oligopeptidase) family of proteases is an enzyme family with the ability to cleave peptides at internal proline residues. As such, this enzyme class has been extensively investigated as poten- tial pharmaceutical targets for neurological diseases [4]. The serine protease sub-family of PEPs (EC 3.4.21.26) is structurally and functionally related to the dipeptidyl pep- tidase IV (EC 3.4.14.5), oligopeptidase B (EC 3.4.21.83) and acylaminoacyl peptidase (EC 3.4.19.1) sub-families, as all of these enzyme sub-families are members of the S9 peptidase family [5].

The objective of this review is to describe the structure and function of PEP enzymes and how they are currently being evaluated as drug targets or as drug candidates themselves. PEP activity was first reported in 1971 af- ter the observation of the cleavage of the prolyl-leucyl peptide bond of oxytocin in homogenates of the hu- man uterus [6]. Since this discovery, PEP enzymes have been isolated from archaeal, eubacterial, and eukaryotic sources [5]. The structure of the first PEP, a serine pro- tease, was solved in 1998, demonstrating a two-domain structure with a unique seven-blade β-propeller domain [7]. The properties of this seven-blade β-propeller do- main have been extensively studied with respect to filter- ing incoming peptides and cleaving them in concert with the peptidase domain. The findings of recent experimen- tal, simulation, and X-ray crystallographic studies will be one focus of this review. By definition, PEPs hydrolyze the peptide bond on the carboxyl side of internal proline residues. The subsite specificity, chain length restriction, and sensitivity of the enzyme to various conditions, such as pH, ionic strength, and temperature, will be reviewed

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with emphasis on the wide-ranging properties of these enzymes.

PEP activity has been found throughout the body, with a high concentration within the brain [8, 9]. This finding, along with the fact that several neuropeptides, with internal proline residues, are recognized by PEP [10, 11], has led to a significant amount of research on the effects of PEP inhibitors and neurological diseases. PEP activity has been linked to a variety of neurological disorders, such as Alzheimer's disease, amnesia, depression, and schizophrenia, as well as other diseases such as blood pressure regulation, anorexia, bulimia nervosa, and Chagas' disease (*Trypanosoma cruzi*) [4]. The status of the research associated with a representative PEP-specific inhibitor, S 17092, and various neurological disorders is reviewed. PEP has also been studied as a potential therapeutic agent for the treatment of celiac sprue, an inflammatory disease of the small intestine that is triggered by dietary, proline-rich gluten. The status of this drug development effort and the associated challenges are an additional focus of this review paper.

Structure

The first PEP whose crystal structure was solved was the porcine PEP [7]. Since then, the structures of two homologous bacterial PEPs, *Myxococcus xanthus* (MX PEP) and *Sphingomonas capsulata* (SC PEP) [12], have also been solved. Members of this sub-family of PEPs are approximately 75 kDa in size, roughly three times the mass of trypsin and chymotrypsin. These proteins consist of two main domains, the catalytic domain with an α/β hydrolase fold and the propeller domain (Fig. 1). It has a cylindrical shape and is approximately 60 Å in height and 50 Å in diameter. The catalytic domain (residues 1–72 and 428–710; numbering throughout the paper is based on the porcine PEP) includes the canonical catalytic triad comprised of Ser554, His680, and Asp641, and is covalently connected to the propeller domain (residues 73–427) [7].

The catalytic triad (Ser, Asp, His) is located in a large cavity at the interface of the two domains. The serine residue is located on what is called a 'nucleophile elbow,' at the tip of a very sharp turn. It is surrounded by several small residues that provide relatively little steric hindrance [7]. The substrate-binding pocket was mapped by studying PEP complexed with Z-Pro-prolinal, a transition state analog inhibitor. The specificity pocket S1 ensures a hydrophobic environment and is an excellent fit for the proline residue. This specificity is improved by the ring stacking between the proline residue of the substrate (or inhibitor) and the indole ring of Trp595 [7].

The sequence similarity between the peptidase and propeller domains of 28 PEP sequences has been determined. The peptidase domain (residues 1–72 and 428–710) is more conserved than the β-propeller domain. In the peptidase domain, 65 residues (18%) were universally conserved, whereas the β-propeller domain had only 7 (2%) conserved residues. As might be expected, the conserved residues include the catalytic triad as well as residues involved with substrate binding. In particular, the S1 specificity pocket was highly conserved, while the S2 and S3 specificity pockets were more variable [5].

A characteristic structural component of PEP is its β-propeller domain (Fig. 1). The domain consists of residues
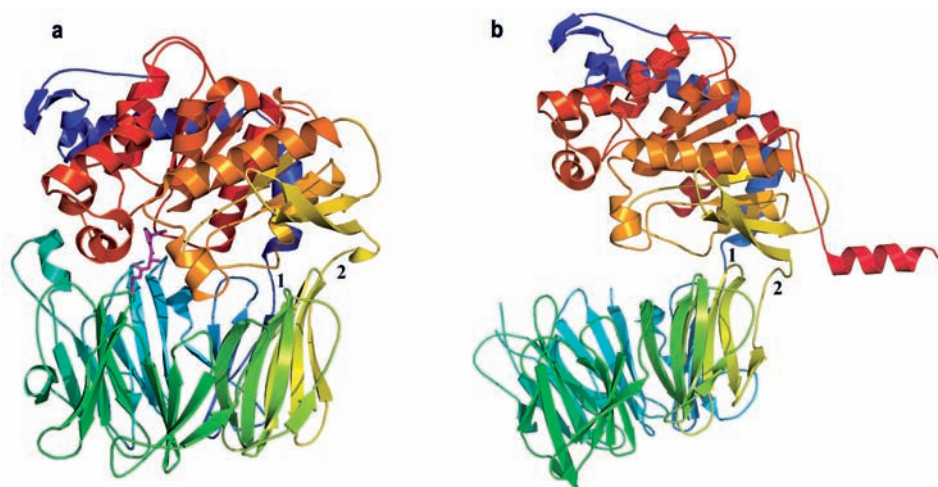

Figure 1. Tertiary structures of *Myxococcus xanthus* (MX) and *Sphingomonas capsulata* (SC) prolyl endopeptidases (PEPs) [drawn and rendered with PYMOL (Delano Scientific, San Carlos, Calif.)]. (*a*) Side view of inhibitor-bound MX PEP, represented by ribbon diagrams. The bound inhibitor Z-Ala-prolinal is colored in magenta. The inhibitor interacts only with the front half of the propeller domain. The two domains are covalently connected by two linkers consisting of residues 67–70 (marked as 1) and 407–409 (marked as 2). (*b*) Side view of the open SC structure. The two connecting strands between the domains are numbered as 1 (Ile106 ~ Glu109, in blue) and 2 (Thr453 ~Pro449, in yellow). The C-terminal His$_6$-tag is fitted into an α helix trailing on the right, in red. [From Shan et al. [12] with permission.]

73–427 and is comprised of a seven-fold repeat of four-stranded antiparallel β sheets. The sheets are twisted and arranged radially around a central tunnel. Typically, with β-propeller domains, ring closure is achieved by constructing the last blade of the propeller domain out of four antiparallel β strands derived from both termini (termed molecular 'Velcro'). Alternatively, in four-bladed β-propeller domains, the structure is stabilized by disulfide bonds between the first and last blades [13]. Neither of these architectural motifs is observed in PEPs. The β-propeller domain of a PEP is only held together by hydrophobic interactions between the first and last blades [7]. Additional stabilization is presumably derived by connecting both ends of the polypeptide chain comprising the propeller domain to the same (catalytic) domain [13].

The catalytic residues of the structurally characterized porcine PEP are buried at the domain interface. This has presented a conceptual challenge for understanding how relatively large peptide substrates gain access to the active site. The observations that the β-propeller domain has a central tunnel, partially covered by flexible side chains, and that it lacks the 'Velcro' seal led to an initial proposal that peptides could enter the active site through the central tunnel of the β-propeller domain. However, the tunnel exit (4 Å in a resting state) is not wide enough to permit typical peptides (6–12 Å in diameter) to enter. It was proposed that this tunnel entrance could enlarge via movement of flexible side chains, which in turn would be facilitated by the opening of the propeller between the first and seventh blades. This hypothesis could also explain how the enzyme excluded large structured peptides and proteins from the active site [7, 14]. More recently, however, studies on the PEP from the hyperthermophilic archeon *Pyrococcus furiosus* led to an alternative theory for substrate regulation and active site access. The *P. furiosus* PEP demonstrated the ability to cleave azocasein as well as the ability to hydrolyze itself [15]. These observations led to the hypothesis that substrates access the active site via a gap between the two domains which can be enlarged by the 'hinge' connecting the domains [16]. More recent experiments have demonstrated that *P. furiosus* PEP does not display significant activity against azocasein and that the autolysis is likely an experimental artifact [17].

Recent experiments have provided further support for the interdomain substrate access hypothesis. PEP mutants, in which disulfide bonds were engineered to force the peptidase and propeller domains into a closed position, destroyed the catalytic activity of the enzyme and eliminated substrate binding [18]. When expressed as an independent entity, the seven-blade propeller domain had higher structural stability than the parent PEP, suggesting that the catalytic domain destabilizes the propeller domain and facilitates concerted movement between the two domains [19]. A molecular dynamics and molecular framework analysis of the flexibility of the PEP protein was also performed to determine the likely areas of movement. The analysis identified a substrate access site between the two domains, whose movement was facilitated by a flexible hydrophilic loop from the propeller domain (residues 192–205) and the N-terminal segment of the peptidase domain [20]. Perhaps most directly, the X-ray crystal structure of a bacterial PEP (*S. capsulata*) was recently solved, with the enzyme in its open conformation (Fig. 1). The structure shows an asymmetric opening between the two domains, with one side separated by about 30 Å and the other side of the two domains still interacting with each other [12].

Structural analysis of the SC PEP, in conjunction with the X-ray crystal structure of the closed form of MX PEP and kinetic analysis of selected MX PEP mutants, led to the following proposed mechanism for substrate binding. The incoming peptide substrate induces a conformation change in the enzyme, causing the domain interface to open. The open structure is temporarily stabilized by interactions between the catalytic domain and the substrate. The extent of the substrate-catalytic domain interaction depends on the size of the peptide. Residues from the catalytic domain mouth area serve as anchors for longer substrates, while other key residues (such as Arg572 and Ile575 for MX PEP) control opening and closing of the enzyme. Additional residues in the propeller domain are important for stabilizing the closed (unbound) structure, but do not appear to interact with the substrate-bound substrate [12].

Function

This section will briefly describe the substrate specificity, pH and ionic strength sensitivity, as well as temperature and proteolytic stability of PEPs. Emphasis will be placed on properties that are markedly different among homologous PEP enzymes.

Specificity

PEP enzymes cleave at internal proline residues within a peptide sequence. The k_{cat}/K_M values for cleavage of a Y-Pro-X construct are 100- to 1000-fold higher than corresponding parameters for the Y-Ala-X bond [21]. The specificity of the S′ subsites (downstream of the scissile bond) of the *Flavobacterium meningosepticum* PEP (FM PEP) was determined by positional scanning via acyl transfer. The S_2' subsite had the highest specificity of the S_1'–S_3' subsites. This subsite preferred Pro residues, followed by Leu, Met, Phe, and Ala residues. The S_1' subsite had lower specificity than the S_2', with the strongest preference for hydrophobic (Leu) and aromatic (Phe) residues, and the greatest discrimination against Pro residues [22].

The majority of the kinetic studies performed on PEP enzymes have been limited to short, synthetic substrates of 2–6 residues [23–25] or slightly longer neuropeptides of <15 residues with only one or two internal proline residues [26, 27]. The kinetics and specificity against proline-rich substrates of varying lengths were recently investigated for three bacterial PEPs (FM PEP, MX PEP, and SC PEP). Each enzyme was evaluated for its ability to cleave a short, chromogenic substrate (Suc-Ala-Pro-pNA), an intermediate-length peptide (13-mer; PQPQLPYPQPQLP), and a long peptide (33-mer; LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF). For the 13-mer peptide, the FM PEP enzyme preferentially cleaved at the PQPQLPYP↓QPQLP site (cleavage site indicated by the arrow), while the MX PEP enzyme preferentially cleaved at PQPQLP↓YPQPQLP. The SC PEP enzyme had comparable preference for both cleavage sites. Overall, FM PEP preferred Pro-Gln bonds, SC PEP cleaved at both Pro-Gln and Pro-Tyr bonds, and MX PEP showed a slight preference for Pro-(Tyr/Phe) bonds over Pro-Gln sites. With regard to chain length specificity, both SC PEP and MX PEP preferred the 13-mer peptide over the 33-mer peptide, while FM PEP showed comparable specificity for both peptides. As discussed below, both sequence specificity and chain length specificity are important for the use of PEPs as potential therapeutic agents [28].

Until recently, it was generally thought that the chain length specificity of most PEPs is capped at 30 amino acid residues. This conclusion was based on initial studies that showed that PEP could degrade peptides of molecular weights less than 3000, but could not hydrolyze high-molecular-weight proteins, such as albumin or elastin [26, 27]. These findings were supported by the initial X-ray crystal structure of porcine PEP and the hypothesis that the β-propeller domain serves as a gating filter to limit substrate accessibility to the active site. However, recent structural and experimental data demonstrate that the chain length limit for at least some PEP enzymes may be considerably longer. For example, certain PEPs have been shown to digest a 33-mer gluten-derived, proline-rich peptide with a specificity comparable to that shown for shorter peptides [29]. Additionally, we have observed cleavage of intact proteins by bacterial PEPs [unpublished results]. Thus, substrate accessibility to the PEP active site, rather than chain length specificity per se, appears to be the primary factor that limits PEP specificity. This limitation is influenced in turn by a combination of many factors including substrate flexibility, inter-domain dynamics and the PEP 'bite' size.

pH and ionic strength sensitivity

The pH sensitivity of PEPs differs from that of the traditional serine proteases, such as chymotrypsin and subtilisin. With traditional serine proteases, the catalytic histidine residue ($pK_a \sim 7$) governs the pH dependence of the catalysis and conforms to a simple dissociation curve. However, the pH profile for PEP enzyme activity is typically a doubly sigmoidal curve, with peaks at pH 6 and 8 and minimal activity outside the pH range 5–9. The doubly sigmoidal curve occurs because PEPs have two catalytic forms that interconvert in a pH-dependent fashion, with one form dominant at pH 6 and the other form dominant at pH 8 [30, 31]. The low-pH form of the enzyme exhibits a significant kinetic deuterium isotope effect, as typically observed for serine proteases, indicating that general base/acid-catalyzed acylation is rate limiting. However, the high-pH form, which is the predominant physiological form, shows essentially no kinetic deuterium effect, suggesting that a conformation change in the enzyme dominates the reaction in the physiological form [30]. Intrinsic fluorescence measurements have been used to evaluate the structural differences between these two forms, and have shown that the low-pH form is more unfolded [32].

Unlike other serine proteases such as chymotrypsin and subtilisin, PEP activity is strongly influenced by ionic strength. Increasing levels of sodium chloride, up to 2 M, revealed an approximately linear increase in the second-order rate constant of the enzyme. This rate enhancement was more pronounced at pH 8.0 than at pH 6.0. Similar enhancements in activity were observed for potassium, phosphate, and nitrate ions [30].

Thermal and proteolytic stability

PEPs have a wide range of thermal stability. A 71-kDa thermophilic PEP has been isolated from the hyperthermophilic archeon *P. furiosus*. This enzyme has optimum activity between 85 °C and 90 °C. It is stable at 4 °C and 37 °C for 24 h, and has been shown to maintain activity overnight at 85 °C [15, 17]. Interestingly, *P furiosus* PEP is homologous to both FM PEP (30% identity, 52% similarity) and the mammalian porcine PEP (32% identity, 54% similarity) [33], even though the latter two enzymes have vastly different optimum temperatures than the *P. furiosus* PEP [16].

The thermal stability of FM PEP has been extensively investigated as a prototype for assessing the potential of engineering a thermostable PEP. The practical advantage of a thermally stable PEP is that the enzyme could be used as a catalyst in peptide-coupling reactions for pharmaceutical production [34]. By using directed evolution techniques, the thermostability of FM PEP was improved by 1200-fold as measured by the $T_{1/2}$ of the enzyme at 60 °C. The thermostable FM PEP contained 12 individual mutations, many of which were located near the N and C termini of the enzyme [34, 35].

The proteolytic stability of PEPs has also been investigated. Limited proteolysis of porcine PEP with trypsin re-

vealed a cleavage site at the Lys196-Ser197 bond, which was later determined to be in the flexible loop region of the propeller domain that interacts with the catalytic domain. Remarkably, the 'nicked' enzyme resulting from limited trypsin digestion was more active than the native enzyme under low-pH conditions [36]. Molecular dynamics analysis confirmed that this loop region of the enzyme (residues 192–205) has the highest flexibility within the enzyme [20]. The ionic strength of the buffer also greatly impacted proteolytic susceptibility of PEP, as the rate of proteolytic breakdown was decreased in the presence of 0.5 M NaCl [36].

The stability of two bacterial PEPs (FM PEP and MX PEP) to the pancreatic enzymes, trypsin, chymotrypsin, elastase, and carboxypeptidase A, was evaluated at pH 6.5. The activities of both enzymes were remarkably stable, as greater than 60% of the initial activity remained after a 60-min incubation time at 37 °C [28]. In addition, MX PEP was demonstrated to retain a vast majority of its activity when incubated with trypsin and chymotrypsin at levels as high as 3 mg/ml for 60 min [37]. It should be noted that in both of these studies, enzyme stability was determined by measuring activity changes. As such, it was not determined if the enzyme remained intact or was cleaved into active 'nicked' enzyme forms.

The discovery and characterization of the highly active 'nicked' enzyme form of porcine PEP raises several interesting possibilities. First, the highly flexible loop region (residues 192–205 in the porcine PEP) is a prime candidate for protein engineering to improve the inherent proteolytic stability of PEP. Second, the improved k_{cat}/K_m of the 'nicked' enzyme, which has a molecular weight of approximately 51 kDa, leads to the idea of creating a peptidase domain-only enzyme. This idea, which was initially proposed by Fulop et al. [7], is supported by the fact that the catalytic triad and the S1 and S2 subsites are located in the peptidase domain. However, attempts to express the catalytic domain of PEP in isolation yielded insoluble protein [19]. A single-domain PEP enzyme may facilitate improved access to large substrates such as intact proteins and would be of potential benefit as a therapeutic agent for treating celiac sprue (see below). For this, critical propeller domain residues required for binding and anchoring the substrate need to be fully identified and potentially added to loop sections in the peptidase domain.

Physiology

In mammals, three different types of PEP enzyme have been reported: cytoplasmic [38], serum [39], and membrane [40]. This section will focus solely on the cytoplasmic PEP, as its biology has been most extensively studied. (Indeed, the identities of the serum and membrane forms of PEP are currently unclear.) The precise physiological role of PEP is not yet known.

Distribution

The distribution of PEP in humans and rodents (rats and mice) has been evaluated at both the tissue/organ level and the subcellular level. Both perspectives are critical for understanding the biology of PEP and for rational drug design. Most localization experiments have been performed by measuring enzyme activity in various samples, although the origin of this activity has not been positively identified as PEP. Typically, activity is measured using a chromogenic substrate, such as Suc-Gly-Pro-MCA [8] or Z-Gly-Pro-β-naphthylamide [9]. In some studies, the loss of activity when adding a known PEP-specific inhibitor, such as Z-thio-proline-thioprolinal, is confirmed [41].

PEP activity has been found throughout the mammalian body and is especially high in the testis, liver, lung, skeletal muscle and brain [42]. Recent studies on rat tissues have shown that PEP activity is approximately two to three times higher in brain than in other tissues (such as the lung, heart, liver) [9]. For human fluids, activity was detected in semen (highest of the fluids), serum, and saliva, but not in the urine [42]. Studies performed on human tissues have shown that PEP activity was present in all tissues [8, 41], but significantly lower levels were present in fluids [41]. The highest level of activity was in the muscle, testes, kidney, and submandibular gland [8]. PEP activity has also been found elevated in human tumor tissues [41].

As PEP has been demonstrated to cleave neuropeptides *in vitro*, the distribution of PEP in the human and rat brain has been studied [8, 9]. PEP activity in both soluble and particulate fractions of postmortem human and rat brain samples has been determined. In the human brain, the highest PEP activity was found in the brain cortex, and the lowest activity was found in the cerebellum. In the rat brain, PEP activity was more homogeneous, with the highest level in the amygdala. Overall, the level of PEP was significantly higher in the human than in the rat brain [9]. The distribution of PEP mRNA has also been determined in the central nervous system and pituitary of the rat, using quantitative reverse transcriptase-polymerase chain reaction analysis and *in situ* hybridization histochemistry. The results demonstrated that the PEP mRNA distribution overlapped with that of various neuropeptide receptors, suggesting the PEP is involved with the inactivation of regulatory neuropeptides [43].

The subcellular distribution of PEP could be critical for understanding the physiological role of the enzyme. Soluble and particulate PEP activity distribution was measured for both human and rat brain samples by a differential centrifugation method. PEP activity was present in all subcellular locations for both human and rat brain

samples. The highest concentration of soluble PEP activity was found in the cytosol. However, for particulate PEP activity, the highest levels were found in the synaptosomal and the myelin fractions. The subcellular distribution of PEP activity was similar for both human and rat brain samples [9].

The subcellular localization of PEP was determined in human glioma and neuroblastoma cell lines by three different methods. First, by using differential centrifugation complemented with Western blot and enzyme activity analysis, the vast majority of PEP was found to be located in the soluble cytosolic fraction. Furthermore, a lactate dehydrogenase assay demonstrated that any extracellular PEP activity is a result of cell lysis, and not protein secretion. Immunocytochemistry analysis (using the monoclonal antibody 4D4D6) showed a fibrillary, cytoskeleton-like distribution of PEP. The PEP immunoreactivity was displayed primarily in the cytosol, with a high concentration in the perinuclear space. These results were confirmed by the intracellular distribution of a PEP-enhanced green fluorescent protein fusion protein. Additional experiments determined that PEP is co-localized with tubulin and is a binding partner of tubulin [44].

Function

As mentioned previously, the precise physiological function of PEP is unknown. The activity of PEP in hydrolyzing bioactive neuropeptides, such as substance P and arginine-vasopressin, *in vitro* [10, 11] led to various studies correlating PEP activity and its inhibition with learning, memory, and psychiatric disorders [4]. PEP inhibition has also been correlated to increased levels of neuropeptides in serum and brain samples (see below). However, PEP is primarily located in the cytosol, while the interactions between neuropeptides and their receptors occur on the cell surface [45]. These data indicate that PEP is involved in the regulation of neuropeptide levels in the body, although the mechanism remains unknown. Recent studies have investigated potential cytosolic functions for the PEP enzymes.

One hypothesis for the mechanism of PEP with respect to mood stabilization, learning, and memory is that PEP is linked to the control of inositol, which plays an important role as a cellular second messenger. Three mood-stabilizing drugs (lithium, carbamazepine, and valproic acid) have been shown to inhibit the collapse of sensory neuron growth cones and increase growth cone area. Inositol has been shown to reverse the effect of these drugs on growth cones. In addition, when studying the development of *Dictyostelium*, which is sensitive to both lithium and valproic acid, it was shown that a PEP knockout mutant as well as PEP inhibitors reverse the effect of the drugs [46].

The precise mechanism through which PEP regulates inositol concentration is not understood. It was demonstrated that the PEP knockout strain had elevated levels of multiple inositol polyphosphate phosphatase (MIPP) activity. MIPP controls the breakdown of higher-order inositol phosphates to inositol $(1,4,5)$ trisphosphate $(InsP_3)$. This finding suggests that PEP presumably regulates the activity of MIPP via the cleavage of a proline-containing oligopeptide [45, 47, 48].

Another potential physiological function of PEP was suggested based on the intracellular location of PEP and its relation to tubulin. Co-localization of PEP with tubulin suggests that PEP may be involved in microtubule-associated processes, such as intracellular trafficking, sorting, and protein secretion. To test this hypothesis, overall protein/peptide secretion was measured in human glioma cells with PEP inhibition or PEP antisense mRNA expression. The reduction in PEP activity was correlated to an increase in protein secretion, suggesting that PEP may be involved in regulating secretory processes [44].

Other proline-specific endopeptidases

In addition to the serine protease subclass of PEPs, several other enzymes with cleavage specificity for internal proline residues have been reported in the literature. These include a metalloproteinase from *Penicillium citrinum*, a peptidase from *Lactobacillus helveticus*, a protease from *Aspergillus niger*, and a peptidase from bovine serum.

The metalloendoproteinase from *P. citrinum* belongs to the family of M35 proteases. This enzyme, named penicillolysin, was characterized against a variety of substrates, including proline-containing peptides such as substance P, dynorphin-A, and neurotensin. It cleaved these substrates at a variety of P_1 residues, including proline and arginine, as well as hydrophobic residues (tryptophan, phenylalanine, tyrosine, and leucine) [49–51]. However, we were unable to detect activity of this enzyme against proline-rich gluten peptides [unpublished data], suggesting that the enzyme has other determinants of substrate specificity at the primary- or secondary-structure level.

Endopeptidases from *L. helveticus* CNRZ32 were characterized for their ability to cleave proline-containing peptides, β-casein and α_{S1}-casein, associated with bitter taste in cheese. Post-proline cleaving ability was detected for three enzymes (PepO2, PepO3, and PepF), PepO2 having the highest activity. Based on sequence comparison, PepO2 and PepO3 were also predicted to be metalloendopeptidases [52, 53]. Recombinant *L. helveticus* PepO2, cloned and expressed in *Escherichia coli*, did not reveal activity against proline-rich, gluten-derived oligopeptides. It was also unable to cleave short chromogenic substrates such as Z-Gly-Pro-pNA [unpublished results].

The prolyl endoprotease from *A. niger* was initially characterized for its ability to debitter beer [54, 55]. Based on sequence comparison, the *A. niger*-derived enzyme belongs to the S28 family of serine proteases, rather than the S9 family to which PEPs belong [54]. The pH optimum of this enzyme is around 4–5, which differs from the neutral pH optimum of the two-domain PEPs described above. Recently, the activity of the *A. niger* enzyme against a variety of gluten-derived, proline-rich peptides has been reported [56]. Based on its reported ability to cleave gluten peptides under simulated gastric conditions, the enzyme may represent a promising tool for celiac sprue therapy (see below).

An enzyme that cleaves the PEP substrate Z-Gly-Pro-MCA but is not inhibited by the PEP-specific inhibitor Z-Pro-prolinal has been isolated from bovine serum. This enzyme, initially named ZIP (Z-Pro-prolinal insensitive peptidase), was further purified, sequenced, and determined to have homology to a seprase (or, fibroblast activation protein) enzyme. The optimum pH profile for this enzyme is similar to that of PEP; however, ZIP differs from PEP with respect to inhibitor susceptibility, and initial investigations indicate that ZIP is more specific than PEP [57, 58].

Potential therapies and disease treatments

PEPs have been investigated as potential therapeutic targets as well as therapeutic agents. Since PEP activity, especially in the serum, has been correlated to neurological disorders such as depression, mania, schizophrenia, anxiety, anorexia, and bulimia nervosa [59–61], there has been an active search for PEP-specific inhibitors. Both irreversible and reversible inhibitors have been developed. In general, irreversible inhibitors contain either a chloroacetyl or a diazo acetyl moiety, which covalently binds to the enzyme. Reversible inhibitors typically contain a formyl group that reacts with the catalytic serine [62]. The pharmacological status of the PEP inhibitor S 17092 in various neurological disorders is discussed below as an example. Over the past several years, orally administered PEP has been investigated as a potential therapeutic agent for the treatment of celiac sprue, a multi-factorial disease characterized by an intestinal inflammatory response to ingested, proline-rich gluten. The status of this ongoing research is also presented below.

PEP inhibitor S 17092

The current literature includes detailed studies of the effects of this inhibitor on specific neuropeptide levels in animals [63]. In addition, a phase I human study has been reported. S 17092 has a high affinity for human PEP, with a K_i of 1 nM [64]. The inhibitor was demonstrated to be selective for PEP, as it was shown not to inhibit related known activities found in human brain homogenate. S 17092 was also shown to be able to penetrate into a human cell line (HEK293, a model for Alzheimer's disease) and inhibit the intracellular activity of PEP with a K_i of 30 nM [64].

The effects of the S 17092 inhibitor on PEP activity levels and specific neuropeptide levels in the rat brain have been studied. A dose of 10 mg/kg of S 17092 reduced the activity of PEP in the rat medulla oblongata by 78% (for a single dose) and by 75% (after a chronic oral treatment). A single administration of S 17092 (30 mg/kg) was able to cause a significant increase in substance P- and α-melanocyte-stimulating hormone-like immunoreactivity in the frontal cortex and hypothalamus. However, this effect was not seen for the chronic administration of the inhibitor [65]. An analogous study was performed to evaluate the effect of S 17092 on two other neuropeptides, thyrotrophin-releasing hormone (TRH) and arginine-vasopressin (AVP). A single oral administration of S 17092 at doses of 10 or 30 mg/kg significantly increased the levels of TRH-like immunoreactivity in the cerebral cortex and of AVP-like immunoreactivity in the hippocampus. Chronic treatment with S 17092 also showed a significant increase of TRH-like immunoreactivity levels in the cerebral cortex [66]. These results show the potential use of PEP inhibitors in the regulation of neuropeptide levels in particular areas of the brain.

Studies have been performed in both rodents and monkeys to determine the effect of S 17092 administration on various mental functions. Specifically, the enhancement of memory performance and/or prevention of memory deficits were evaluated in two memory-based tasks in mice. At doses of 10 mg/kg twice daily for 7 days, S 17092 facilitated learning function related to both work (delayed alternation test) and reference (spatial discrimination test) memory [67]. The ability of S 17092 to improve cognitive deficits was also examined in monkeys treated with chronic, low-dose 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, a model of early stage Parkinson's disease. The chronic administration of S 17092 over a 7-day period was able to improve the performance of the animals in several cognitive tasks. The PEP inhibitor was most efficacious at a dose of 3 mg/kg, showing no effect at a lower dose of 1 mg/kg. S 17092 showed no efficacy in cognitive performance tests after 3 days of administration [68].

A phase I study on the pharmacodynamics and pharmacokinetics of S 17092 was performed in healthy, elderly volunteers. Four doses (100, 400, 800, and 1200 mg) were investigated in sequential order. Each patient (36 in total) was administered a single oral dose on day 1, and then, after a 1-week washout period, was given a single oral dose daily for 7 days. Plasma PEP activity, quantitative electroencephalogram (EEG), and psychometric tests were used to determine pharmacodynamics. Overall, S
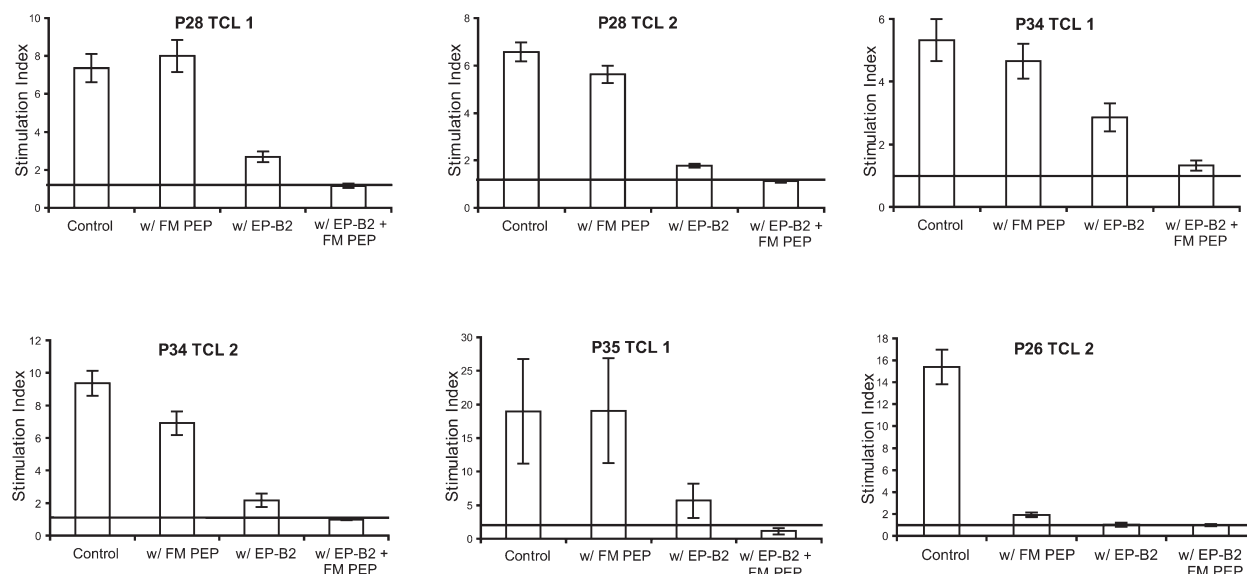
Figure 2. Detoxification of food-grade gluten using the two-enzyme glutenase. Gluten was digested with pepsin alone (P) or pepsin supplemented with EP-B2 (25 : 1 gluten : EP-B2) for 60 min, followed by treatment with trypsin, chymotrypsin, elastase, and carboxypeptidase A (TCEC) with or without FM PEP (75 : 1 gluten : FM PEP) for 10 min. The antigen content of these digests was measured using T cell proliferation assays, with a stimulation index of 1 (denoted with a horizontal line) indicating background levels of proliferation. The control represents gluten that has been digested by pepsin and the pancreatic enzymes (TCEC) without EP-B2 or PEP. The name of individual T cell lines is presented at the top of each graph (e.g. P28 TCL 1). From Siegel et al. [81] with permission.

17092 was well tolerated by all patients and demonstrated a long-lasting, dose-dependent inhibition of plasma PEP activity. However, the EEG and psychometric tests were inconclusive, as EEG results showed marked variability, and the two tests indicated different effective doses. A limitation of this study was that it did not establish whether inhibition of plasma PEP could be correlated to a reduced concentration of critical neuropeptides, such as substance P or vasopressin [69].

The concept of targeting PEP with specific inhibitors to control critical neuropeptide levels in the body is an intriguing one. However, a better understanding of the physiological function of PEP and its specific mechanism for regulating neuropeptide levels is critical to design appropriate inhibitors that target the proper region of the body.

PEP as a therapeutic agent – celiac sprue

Celiac sprue, or celiac disease, is a high-prevalence, heritable disorder of the small intestine that affects both children and adults (1 : 100–1 : 300) [70, 71]. This multifactorial disease is characterized by an inflammatory response to ingested wheat gluten and similar rye and barley proteins, leading to damage of the intestinal villi and a greatly reduced ability to absorb nutrients [72–74]. If left untreated, celiac sprue is associated with increased risk of anemia, infertility, osteoporosis, and intestinal lymphoma. A gluten-free diet is the only accepted treatment for celiac disease. However, complete exclusion of di-

etary gluten is very difficult due to the ubiquitous nature of this protein, cross-contamination of foods, inadequate food labeling regulations, and social constraints [75].

Obtained from a grocery store, wheat gluten is a complex protein consisting of a mixture of many gliadin and glutenin polypeptides. Interestingly, gluten proteins are rich in proline (~15%) and glutamine (~35%) residues, a feature that is especially notable among gluten epitopes that are recognized by disease-specific T cells [74]. In fact, proline occupies four of the nine positions in the core region for several immunodominant epitopes [74]. The high proline concentration makes these inflammatory T cell epitopes relatively resistant to digestion by pancreatic enzymes. When proteolyzed by pepsin and pancreatic enzymes under physiological conditions, dietary gluten yields a complex mixture of thousands of peptides of varying lengths. Mass spectrometry analysis of the proteolyzed gluten revealed epitope-containing peptides of varying lengths, with 40% from 11–20 residues (27 of 68), 46% from 21–30 residues (31 of 68), and 15% longer than 30 residues (10 of 68) [76]. One peptide, a multivalent 33-mer peptide (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) generated from an α-gliadin protein under physiological conditions, is an exceptionally potent pro-inflammatory sequence that is recognized by mucosal T cells found in all celiac sprue patients tested thus far [29].

PEPs are attractive drug candidates for the oral treatment of celiac sprue due to their ability to accelerate gluten digestion in the gastrointestinal tract. A variety of assays, including high-performance liquid chromatography, mass

spectrometry, T cell assays, anti-gluten assays and even gluten challenge studies in patients, have been deployed to quantify proteolytic gluten detoxification [12, 28, 29, 37, 76–80]. PEPs with an acidic pH profile, such as the *A. niger* protease, could be used to accelerate gluten breakdown in the stomach [56]. At the same time, PEPs with optimal activity around neutral pH (e.g. FM PEP, MX PEP, or SC PEP) would be effective in detoxifying gluten in the upper small intestine, where a bulk of protein digestion and absorption occurs. If the latter type of therapy is to be efficacious, the enzyme must be enteric coated so that it is protected from the gastric environment and then activated when food is released into the duodenum. A suitable polymer-coated formulation has been developed for MX PEP [37]. Additionally, whereas a gastric PEP must be resistant to pepsin, a duodenal PEP must be sufficiently stable in the presence of pancreatic and intestinal brush border membrane enzymes. Studies have shown that the *A. niger* PEP is resistant to pepsin [56], whereas FM PEP and MX PEP meet the latter criterion [28]. A particularly encouraging form of two-enzyme therapy involves combination of a duodenally active PEP with a glutamine-specific protease that hydrolyzes gluten under gastric conditions [81]. Such a combination therapy was capable of rapidly rendering high loads of gluten completely non-toxic under conditions where no individual enzyme was entirely effective (Fig. 2).

Conclusion

The PEP class of enzymes represents an excellent opportunity as both a pharmaceutical target and agent. Currently, PEP-specific inhibitors are being evaluated for the treatment of a wide variety of diseases, in particular neurological diseases. However, a more detailed understanding of the physiological role of PEP is needed to facilitate this endeavor. Over the past few years, PEP has also been studied as a therapeutic agent for celiac sprue, an inflammatory disease of the small intestine triggered by proline-rich gluten. *In vitro* and *in vivo* results have set the stage for controlled clinical trials. In each case the structure and mechanism of PEP enzymes has played a critical role in facilitating pharmacological goals.

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