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Peptide Amidation: Production of Peptide Hormones *in vivo* and *in vitro*

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Abstract Over half of all biologically active peptides and peptide hormones are α-amidated at their C-terminus, which is essential for their full biological activities. Amidation is accomplished through the sequential reaction of the two enzymes encoded by the single bifunctional, peptidylglycine α-amidating monooxygenase (PAM or an α-amidating enzyme). PAM catalyzes the formation of a peptide amide from peptide precursors that include a C-terminal glycine, and requires copper, molecular oxygen, and ascorbate. PAM is the only enzyme that produces peptide amides *in vivo*. However, various strategies utilizing PAM, carboxypeptidase-Y enzymes, and chemical synthesis have been developed for producing peptide amides *in vitro*. The growing need and importance of peptide amide drugs has highlighted the necessity for an efficient *in vitro* amidating system for industrial application. In recent years, recombinant systems for enzymatic amidation have received growing attention for the production of peptide hormones, like calcitonin and oxytocin. This review presents the current situation regarding amidation, with a special emphasis on the industrial production of peptide hormones.

Keywords: amidating enzyme, PAM, PHM, peptide amide, peptide hormone, calcitonin.

INTRODUCTION

Peptides 3 to 40 amino acids long constitute a major group of intercellular agents for cell-to-cell communication, either as messenger hormones or as neurotransmitters and neuromodulators. Peptide hormones and neuropeptides are synthesized as larger proprotein precursors that are cleaved in a sequence-specific and tissue-specific manner to generate biologically active forms. In these processes, several key enzymes are involved. These include signal peptidases, endopeptidases like protein convertases, exopeptidases like carboxypeptidase H (E), and various peptide modification enzymes. Fig. 1 shows general preprohormone processing cascades [1].

Post-translational enzymatic modifications are often essential for the specific recognition of peptides by their cognate receptors, and highly regulated for the synthesis of biologically active peptides [2]. Some peptide hormones require glycosylation for full biological activity. Anterior pituitary hormones, such as the thyroid-stimulating hormone (TSH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH), are the main examples of N-glycosylated bioactive peptides. Acetylation affects the biological activity of peptides either positively or negatively and can increase the *in vivo* half-life of the peptide. The acetylation of the melanocyte-stimulating hormone (α-MSH) and β-endorphin occurs in the secretory granules of the intermediate lobe of the pituitary. The effect is variable: it potentiates the activity of α-MSH, yet abolishes the opiate activity of β-endorphin. Phosphorylation does not seem to play a critical role in the biological activity of peptide hormones. In humans, 33% of the adrenocorticotropin hormone (ACTH) is phosphorylated, whereas 50% and even no phosphorylation is observed in rats and bovine tissue, respectively. Tyrosine sulfation dramatically affects the biological activity of certain neuropeptides. The sulfation of cholecystokinin (CCK) increases peptide effectiveness more than 200-fold and in many cases prolongs the half-life.

Among post-translational modifications, C-terminal α-amidation is known to be the most important and essential for biological activity. This reaction is catalyzed by the bifunctional enzyme, peptidylglycine α-amidating monooxygenase (PAM, EC 1.14.17.3). The importance of the amidation reaction is clear from the observation that about 50% of mammalian peptide hormones and more than 80% of insect hormones have α-amidated C-termini [3]. Representative α-amidated peptides found in nervous and endocrine systems are summarized in Table 1 [4]. Among these, calcitonin, oxytocin, and vasopressin are now widely used for therapeutic purposes. As the therapeutic potential of amidated peptides has gained commercial attention, so the development of an efficient process for amidation in the production of these hormones has grown in importance.

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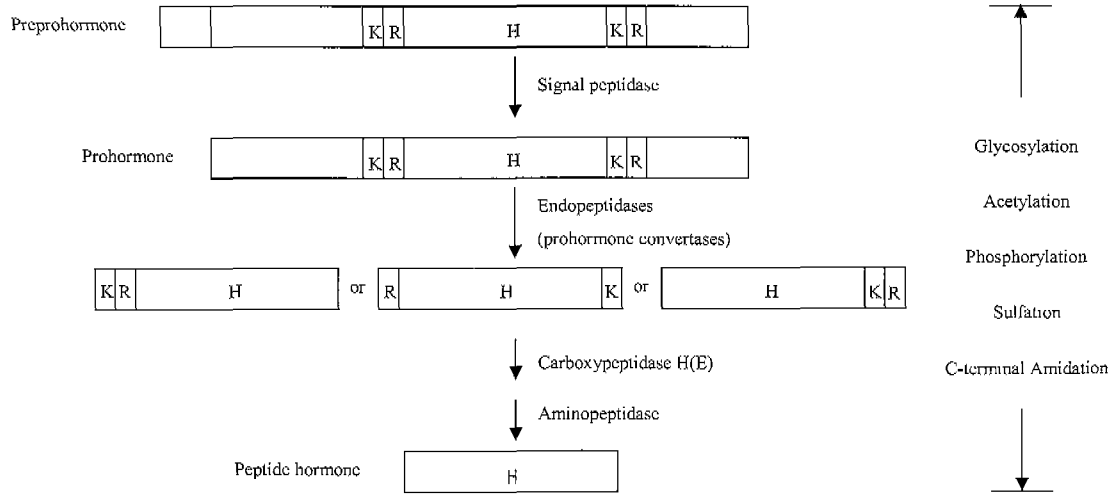


Fig. 1. Preprohormone processing and modification. Active hormones (H, shaded area) are produced *in vivo* through sequential enzyme reactions. In many prohormones, paired basic cleavage sites (K-K, R-R, K-R) are present and undergo endoproteolysis by several prohormone convertases (PCs). Basic residue extensions are removed by carboxypeptidase H (E) and aminopeptidase to yield the active hormone. During these processes, post-translational modifications occur in the secretory granules.

Table 1. α-Amidated peptides found in nervous and endocrine systems

Peptide	Source	Amino acids	C-terminus
Neurokinin A	Mammalian	10	Met-NH_2
Allatostatin	Cockroach	13	Leu-NH_2
Lem-KI	Cockroach	8	Gly-NH_2
TRH	Porcine, Bovine	3	Pro-NH_2
Calcitonin	Human	32	Pro-NH_2
CRF	Bovine	41	Ala-NH_2
LHRH	Porcine	10	Gly-NH_2
Leucopyrokinin	Cockroach	8	Leu-NH_2
Gastrin I	Human	17	Phe-NH_2
Dermorphin	Frog	7	Ser-NH_2
Oxytocin	Mammalian	9	Gly-NH_2
Substance P	Mammalian	11	Met-NH_2
NPY	Porcine	36	Tyr-NH_2
FMRFamide	Clam	4	Phe-NH_2
Bombesin	Frog	14	Met-NH_2
Amylin	Human	37	Tyr-NH_2
Vasopressin	Mammalian	9	Gly-NH_2
Bld-GrTH	Cockroach	10	Thr-NH_2
Gastrin releasing peptide	Human, Porcine	27	Met-NH_2
Neuromedin B	Porcine	10	Met-NH_2
Pancreastatin	Rat	51	Gly-NH_2
Conotoxin M1	Snail	14	Cys-NH_2
Secretin	Porcine	27	Val-NH_2
GHRF	Human	44	Leu-NH_2
Melittin	Honey bee	26	Gln-NH_2
Sarcotoxin 1A	Meat fly	39	Arg-NH_2
VIP	Mammalian	28	Asn-NH_2
α-MSH	Mammalian	13	Val-NH_2
MIF-1	Mammalian	3	Gly-NH_2
Red pigment concentrating factor	Shrimp	8	Trp-NH_2
Pigment dispersing hormone	Shrimp	18	Ala-NH_2

AMIDATION OF PEPTIDES

In vivo Amidation: Structure and Function of PAM

Over 50% of all peptide hormones and neurotransmitters require amidation at their C-terminus to exhibit their full biological activities. This reaction is catalyzed by unique PAM, or an α-amidating enzyme *in vivo*. PAM was first characterized by Bradbury *et al.* [5]. The PAM gene encodes one polypeptide with two different enzyme activityes that catalyze two sequential reactions. The first enzyme, peptidylglycine α-hydroxylating monooxygenase (PHM, EC 1.14.14.3), produces a hydroxylated intermediate from a glycine-extended peptide in the presence of copper, ascorbate, and molecular oxygen. The second enzyme, peptidyl-α-hydroxyglycine α-amidating lyase (PAL, EC 4.3.2.5), converts the peptidyl-α-hydroxyglycine intermediate into an α-amidated peptide and glyoxylate [6-10]. The overall scheme for the two-step reaction catalyzed by PAM is shown in Fig 2 [11]. An interesting feature of this reaction is the origin of the amide group in the product, which comes from the substrate glycine through N-oxidative cleavage, rather than from an amide transfer.

The overall structure of the PAM protein (Fig. 2, bottom) is very similar in many species [12]. The enzyme carries a signal sequence that is removed in the endoplasmic reticulum. The two enzyme domains, PHM and PAL, are separated by an exon A containing an endoproteolytic cleavage site, which can generate soluble PHM and membrane-bound PAL in a tissue-specific manner.

The major expression sites of PAM are the endocrine cells of the pituitary and many neurons. However, the

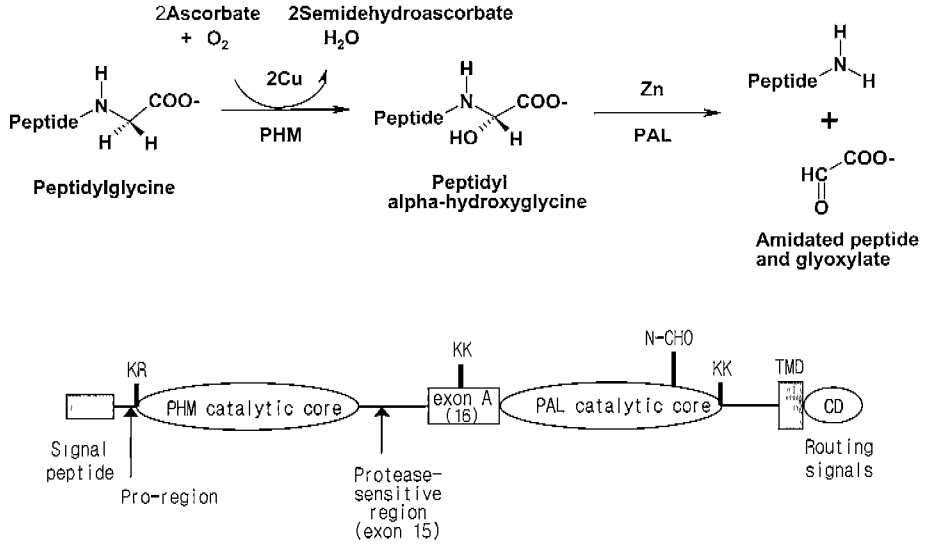


Fig. 2. Two-step PAM reaction and schematic representation of bifunctional membrane PAM protein. A peptidylglycine substrate is converted to an amidated peptide product by the sequential reactions of PHM and PAL (top). Tissue-specific endoproteolytic cleavages between the basic residues or exon 15 region generate several forms of different enzymes (bottom).

expression of PAM has also been detected in several tissues and various cells, including endothelial cells, lung epithelial cells, smooth muscle cells, brain ependymal cells, and astrocytes [13-17]. The crucial role of PAM is exemplified by the fact that a null mutation of the PAM gene results in early embryonic lethality in *Drosophila* [18]. In humans, PAM is the target of drug design for diseases ranging from rheumatoid arthritis to cancer, because the expression level of PAM is abnormal in these tissues [14, 19].

The alternative splicing of mRNA, in a tissue-specific manner, generates at least seven different forms of PAM mRNA in rats [4]. A detailed analysis showed that there are more than 27 exons encompassing over 160kb of the genomic DNA [20]. The proteins encoded by seven different forms of PAM mRNA – three membrane-bound proteins (rPAM-1, 2, 3b) and four soluble proteins (rPAM-3a, 3, 4, 5) – have all been identified. Among these, five proteins (rPAM-1, 2, 3, 3a, 3b) are bifunctional and include both PHM and PAL domains, whereas rPAM-4 carries monofunctional PHM activity and rPAM-5 exhibits no activity. However, the major forms found in the anterior and neurointermediate pituitary of rats comprise only four forms (rPAM-2, 2b, 3, 3a) [21,10].

Husten *et al.* [22] purified and characterized the rat PAM-1 protein from stably transfected hEK-293 cells. Purified PAM-1 exhibits its optimum activity under acidic conditions. Limited tryptic digestion of this integral membrane protein releases a monofunctional PHM domain. This treatment results in an approximate 4-fold increase in the specific activity compared with PAM-1, and a shift in the optimal pH from 4.5 to 5.5. The 35KD tryptic fragment, PHMcc (catalytic core of PHM), previously identified as residue 42 to 356 of the

Fig. 3. Representation of PHMcc structure with dipeptide substrate [23].

PHM domain, has been crystallized in the presence of a substrate and the three-dimensional structure of the cocrystal determined by X-ray diffraction analysis [23]. The structure consists of two domains, domain I and domain II, each containing a copper binding site, separated by about 150 amino acids in length (Fig. 3). Although the two domains contain a similar topological core, they also differ in many ways. Domain I(Fig. 3, right) is composed of 10 β-strands and binds the copper designated as CuA. Domain II (Fig. 3, left) is composed of 12 β-strands and one 3_{10} helix (cylinder in Fig. 3) and binds the copper, CuB. There are three disulfide linkages in domain I and two disulfide bonds in domain II, yet no disulfide bond between domain I and domain II. The two coppers are 11 Å apart facing the interdomain space in such a way that the cleft between them is fully accessible to a solvent.

The proposed reaction mechanism of PHM starts with the reduction of the two coppers from Cu^{2+} to Cu^{+} by two one-electron transfers from two ascorbate molecules [24]. The reduced CuB copper binds molecular oxygen, then a substrate-mediated electron transfer from CuA to CuB occurs with the concomitant reduction of the molecular oxygen. Yet this electron transfer only occurs when a substrate is bound to the enzyme because the distance between the two coppers is too long (11 Å) to transfer an electron in an empty state without a substrate [25]. The reduced oxygen immediately reacts with the bound substrate to produce a hydroxylated product.

Production of Amidated Peptides

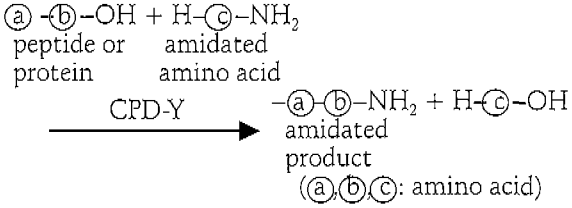
Many pharmaceutical companies and research groups have endeavored to develop an efficient amidating reaction, especially due to its industrial applications in the production of peptide amides for therapeutic purposes. Accordingly, various methods have been developed, including 1) amidation by an α-amidating enzyme from a natural source, 2) amidation by a recombinant α-amidating enzyme, 3) enzymatic transacylation by carboxypeptidase-Y, and 4) chemical synthesis.

Mouse pituitary cells (AtT-20) display an endogenous amidating activity. The cells process and secrete a wide variety of biologically active peptide hormones [26-27]. AtT-20 cells have been stably transfected with the human calcitonin (hCT) gene to secrete hCT in a fully active amidated form, without any extra steps of additional enzymatic or chemical modifications. The expression level was about 17 ng hCT/mL [28]. The non-endocrine cell lines, COS-7 (African green monkey kidney cells with SV40 T-antigen) and CHO (Chinese hamster ovary) cells have also been used to produce salmon calcitonin (sCT) [29]. CHO cells contain substantial amounts of PAM mRNA and PAM enzyme activity, whereas COS-7 cells exhibit a lower activity [30]. Nonetheless, both cells transfected with a calcitonin precursor gene bearing furin cleavage sites produced a similar level of biologically active amidated calcitonin without the coexpression of PAM.

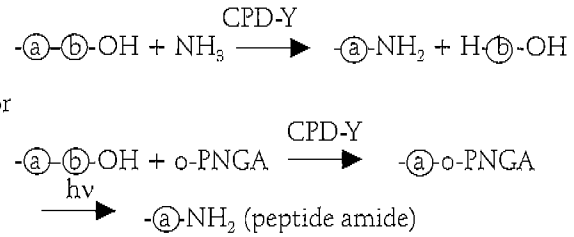
Several research groups have tried to prepare an α-amidating enzyme by recombinant or extraction systems. In *E. coli,* recombinant amidating enzymes have been produced mainly as inclusion bodies. Consequently, refolding processes are required to regain the activity. However, the activity of the *E. coli* derived enzyme was much lower than that of the mammalian host [31-32]. To obtain soluble and active amidating enzymes from *E. coli,* only the PHM or PHMcc domain (without PAL domain) was expressed. Yet the PHM domain was invariably expressed as an insoluble protein. Moreover, the pH had to be raised to the basic condition after the PHM reaction to effect the cleavage reaction resembling that of PAL activity.

Miller *et al.* [33] established a cloned CHO cell line that secretes significant quantities of an active α-amidating enzyme after stable transfection of the rat PAM gene with methotrexate selection. Using this secreted enzyme, Ray *et al.* [34] produced salmon calcitonin by *in vitro* amidation with a peptide precursor obtained from *E.coli.*

Carboxypeptidase-Y (CPD-Y) is a serine carboxypeptidase family, yet it also exhibits transpeptidation activity with a broad specificity of amino acids, including amidated amino acid residues. This is the reason why CPD-Y has been found to be suitable for several applications, *e.g.,* C-terminal sequencing, peptide synthesis, protein semisynthesis, and C-terminal amidation/labeling as follows [35-37].



Nonetheless, the transpeptidation reaction suffers from certain limitations: CPD-Y cannot accept some amino acid amides (proline, glutamic acid, aspartic acid) as nucleophiles. Unfortunately, calcitonin carries proline amide at its C-terminus, therefore, this process cannot produce biologically active calcitonin. To overcome this problem, Henriksen *et al.* [36] designed an approach where a peptide precursor is subjected to a CPD-Y catalyzed transacylation with the photocleavable nucleophile, 2-nitrophenylglycineamide hydrobromide (o-PNGA), followed by photolysis to give the desired peptide amide. This method was found to be more efficient than NH_3 as a nucleophile in transamidation, and the overall yield was higher than 95% [37].



Based on this principle, a growth hormone releasing factor (GHRF, Arg-NH_2 at C-terminus) was obtained [37-39]. Calcitonin was also produced by the sequential reactions of CPD-Y catalyzed transpeptidation and photochemical cleavage [35, 40].

Peptide amides can also be produced by liquid or solid-phase chemical synthesis. C-terminal amidation in the liquid-phase utilizes polyethylene glycol derivatized with an acid-labile or photo-sensitive anchoring group. Immunoregulating peptide amides are produced by solution-phase synthesis [41]. Solid-phase synthesis is a classical method for producing peptide amides, and utilizes acid-cleavable linkers attached to the resins [42]. The most common and easily available linkers are the 4-methylbenzhydrylamine (MBHA) linker [43] and Rink-

amide linker (trialkoxy-diphenyl-methyl ester resin) [44], both of which easily release peptide amides under strong acidic conditions. Eel calcitonin analogues with natural-N-linked oligosaccharides have been synthesized by the MBHA solid-phase method [45]. Adipokinetic hormone-I (AKH-I), a member of red pigment concentrating hormones (Thr-NH_2 at C-terminus), has been obtained by the Rink solid-phase method [46]. Several peptide amides with unnatural side chains have also been synthesized by the solid-phase method [47]. However, these methods are intrinsically limited due to the strong acidic conditions required for the cleavage of the linker groups. Consequently, a peptide that has an acid-susceptible amino acid like tryptophan, cannot be synthesized using these methods. To resolve this problem, Han *et.al.* [48] developed xanthenylamide (XAL) handles for solid-phase peptide amide synthesis under particularly mild conditions. As a result, several peptides, such as the adipokinetic hormone, CCK-8 sulfate (cholecystokinin octapeptide, with Phe-NH_2) and oxytocin (Gly-NH_2) have been synthesized with good yields. Recently, a new aminoethyl-polystyrene linker was developed that allows for the removal of acid-labile protecting groups in a simple and convenient manner [49].

PHM and PAL: Assay Systems and Other Applications

As mentioned above, PAM is a bifunctional enzyme that includes both PHM and PAL activity. Therefore, two different enzyme assays, separate or in concert, are required to monitor the amidation reaction.

PHM catalyzes the stereospecific hydroxylation of the α-carbon of the C-terminal glycine residue and generates peptidyl α-hydroxyglycine. Therefore, short peptides with a terminal glycine extension are commonly used as substrates. To enhance the detection sensitivity, isotope-labeled peptides or chromophore-labeled peptides are often employed. Alternatively, the consumption of oxygen can also be monitored, yet the sensitivity is very low.

PAL generates α-amidated peptides from PHM-catalyzed peptidyl α-hydroxyglycine. The PAL activity can be assayed using hydroxyglycine peptide or α-hydroxyhippuric acid as substrates. The various assays for PHM and PAL are summarized in Table 2, with a special emphasis on their sensitivity and the equipment required [50]. [^{125}I]-Labeled peptide assays and radioimmunoassays (RIA) are the most sensitive, with due precautions and proper handling of the antibodies.

PAM can be a potentially attractive drug target by modulating the synthesis and production of many peptide hormones. Amidated neuropeptides, such as substance P and a calcitonin gene-related peptide (CGRP) turn out to be involved in acute inflammation. 4-Phenyl-3-butenoic acid, an inactivator of PAM inhibited serum PAM activity, lowers endogenous tissue levels of substance P and relieves an acute inflammatory response in an experimental animal model [58].

INDUSTRIAL ASPECT OF BIOACTIVE AMIDATED PEPTIDES

The current worldwide pharmaceutical market size is estimated at more than $250 billion. The therapeutic protein/peptide industry has also grown rapidly and, in 1997, reached over $12 billion, thereby accounting for 5% of the total pharmaceutical market [51].

Since calcitonin is the only amidated therapeutic protein/peptide ranked within the top-five items in the therapeutic protein market (Interferon, Insulin, Human growth hormone, Erythropoietin, and Calcitonin), calcitonin-related industries are the focus of the current discussion. Calcitonin, a 32-amino acid polypeptide amide discovered in 1962, is produced in humans by the parafollicular cells of the thyroid gland. Although it exerts an important role in calcium homeostasis, it is still poorly understood. Calcitonin has been used for many years in the treatment of various metabolic bone diseases, particularly Paget's disease and hypercalcemic shock

Table 2. Comparison of PHM and PAL assays

	Substrate	Sensitivity (pmol)	Equipment and time required
PHM s	[^{125}I]Ac-Tyr-Val-Gly	0.15	γ Counter
	D-[^{125}I]Tyr-Val-Gly	0.15	γ Counter
	Trinitrophenyl-D-Tyr-Val-Gly (TNP-D-Tyr-Val-Gly)	400	HPLC, UV Detector, 8 min/sample
	N-Dansyl-Tyr-Val-Gly	25	HPLC, Fluorescence Detector, 5 min/sample
	Substance P-Gly	0.005	Radioimmunoassay, γ Counter
	Dabsyl-Gly-phe-Gly	1	HPLC, UV Detector, 5 min/sample
	N,N-Dimethyl-1,4-phenylenediamine (DMPD)	500	Spectrophotometer, Continuous assay
	D-Tyr-Val-[^{14}C]Cly [^{14}C]glyoxylate	0.5	Scintillation counter
	4-Nitrohippuric acid	600	HPLC, UV Detector, 8 min/sample
	Oxygen	2500	Oxygen electrode
PAL	[^{125}I]Ac-Tyr-Val-hydroxy-Gly	0.15	γ Counter
	α-OH-hippuric acid	600	HPLC, UV Detector, min/sample

Table 3. Major companies engaged in commercial production of calcitonin

Company	Brand name	Source	Production method
Rhone-Poulenc Rorer	Calcimer	Salmon	Chemical synthesis
Sandoz (Novartis)	®Miacalcin	Salmon	Chemical synthesis
Smithkline Beecham	®Elcatonin	Eel	Chemical synthesis
Ciba-Geigy	®Cibacalcin	Human	Chemical synthesis
Asahi	®Elcitonin	Eel	Chemical synthesis
Suntory	®–	Human	Recombinant PAM
Sankyo	–	Human	Carboxypeptidase-Y
Unigene	–	Salmon	Recombinant PAM

[52]. Until now, considerable clinical attention has been given to calcitonin because of its outstanding role in the prevention and treatment of osteoporosis [53,54].

Salmon calcitonin and eel calcitonin are more potent and have a biological activity 20-40 times greater than human calcitonin. Moreover, whereas the disulfide bond (cys^1–cys^7) in human calcitonin is crucial for its biological activity, it is not so crucial in salmon calcitonin [40]. However, despite the many benefits of salmon calcitonin, it also has certain disadvantages: 1) side effects, like anorexia, nausea, vomiting, a metallic taste, or occasionally an allergic reaction, and 2) the low affinity of a salmon calcitonin precursor for an amidating enzyme [34], which affects the productivity of an *in vitro* amidating reaction. Accordingly, to increase potency and safety, hybrid calcitonins combining the merits from salmon and human sources are under development.

The first commercially available calcitonin was extracted from salmon, yet the current world market is dominated by chemically synthesized products. The companies that share the dividends in the calcitonin market are summarized in Table 3. Suntory Ltd. (Japan) cloned a PAM enzyme from *Xenopus laevis* [55] and produced human calcitonin using a recombinant enzyme. Sankyo Company (Japan) produced human calcitonin by reacting protein substrates including C-terminal prolyl-Leu, prolyl-Ile, prolyl-Val or prolyl-Phe with carboxypeptidase-Y in the presence of ammonia [56]. Unigene Laboratories (USA) is the leading company for the production of recombinant calcitonin. They cloned a PAM enzyme from rat medullary thyroid carcinoma and then expressed it in eukaryotic and prokaryotic systems [57]. Salmon calcitonin has been produced by a combination of a recombinant PAM enzyme and the CNBr cleavage of a CST-fused salmon calcitonin precursor.

CONCLUSION AND PERSPECTIVE

In recent years, interest in PAM has been spurred due to its emerging role in the production of therapeutically important amidated peptides, such as calcitonin, vasopressin, oxytosin, and growth hormone releasing factors *etc*.

The classic chemical synthesis for peptide amidation has many limitations. It is environmentally unfriendly and inefficient. Eukaryotic expression systems for the production of amidated peptides are unsuitable for mass production because they are not as cost-effective as chemical synthesis. To overcome the above-mentioned problems, the current tendency has been to develop efficient procedures for the enzymatic amidation of recombinant peptide precursors. With the improved tools now available, the combination of recombinant procedures and enzymatic amidation can provide alternative means for calcitonin production. Accordingly, several biotechnology companies are currently working on recombinant procedures for amidation.

To obtain active amidating enzymes, recombinant mammalian or *E.coli* cells have been investigated. However, the former cannot produce large enough enzyme quantities to suit industrial needs. While the latter, although facilitating large-scale production, requires a refolding process for the insoluble inclusion body, which remains a technical hurdle. Consequently, the commercial production of peptide amides still requires a recombinant procedure that provides soluble and active amidating enzymes for the routine conversion of peptide precursors to peptide amides.

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