Synthetic peptide protease inhibitors

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American Peptide Company *makes the case for using synthetically-derived protease inhibitors in the manufacture of biological proteins for therapeutic applications*

Yeast, fungal and microbial protein expression systems are used in the production of many therapeutically relevant proteins, such as vaccines, monoclonal antibodies and enzymes. Protease inhibitors can increase production yield with corresponding decreases in the unit cost of manufacturing therapeutic proteins by biological expression.

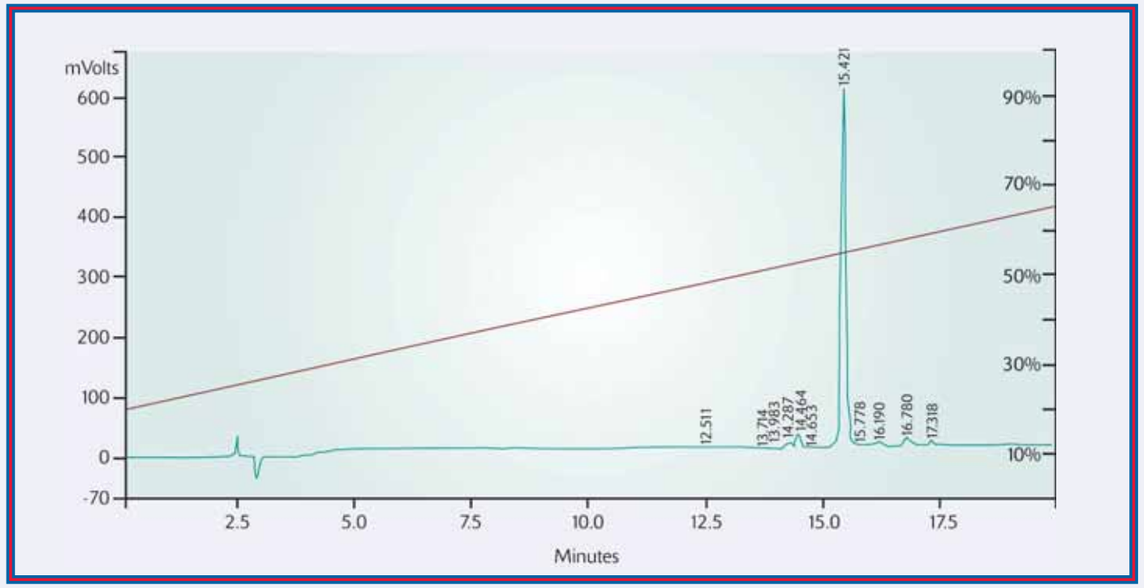
Using modern techniques, peptidic protease inhibitors can be manufactured synthetically at multi-kilogram scales under cGMP conditions to a very high level of purity, yield and activity. Synthetic manufacturing offers advantages in purity, identity and cost over biological sources for selected protease inhibitors.

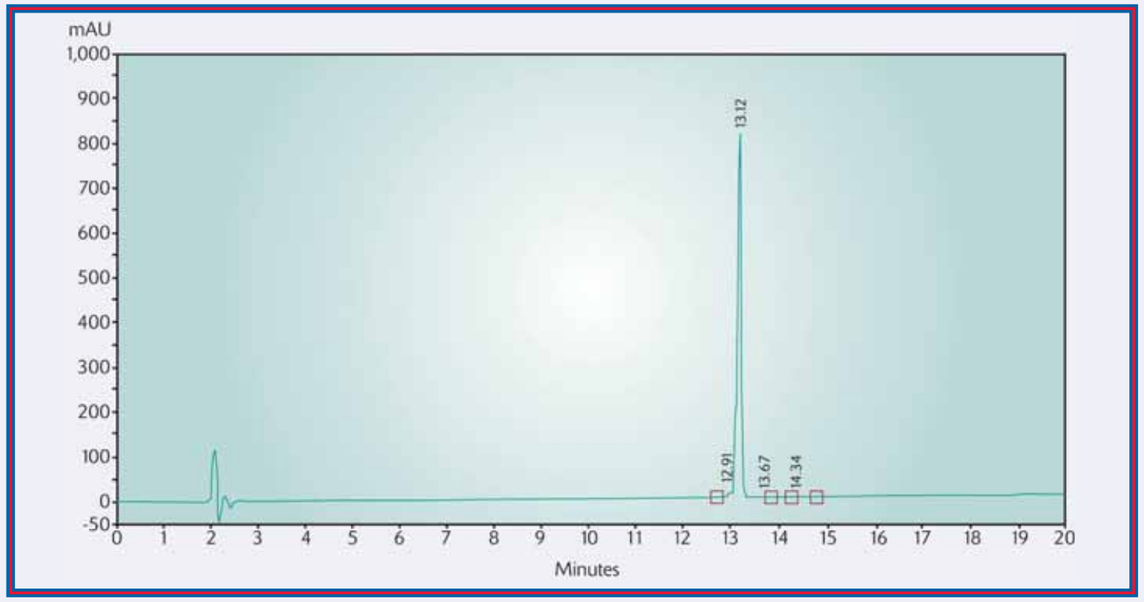
Like their biologically produced analogues, synthetic protease inhibitors can irreversibly modify an essential amino acid of the active site of many proteases to inactivate proteolytic activity. This inhibition confers a beneficial effect on the manufacturing effort by increasing the production yield of the protein of interest with attendant cost improvements. In this paper, two synthetic protease inhibitors are described.

Pepstatin A (MW: 685.9d, CAS: 26305-03-3) is a pentapeptide transition state potent inhibitor of certain microbial aspartic proteases, including cathespsin D, renin, pepsin and bacterial aspartic proteinases, as well as yeast protease A. It was first isolated from culture filtrates of various species of *Actinotomyces.*[1]

The synthetic analogue with a structure Isovaleryl-Val-Val-Sta-Ala-Sta, where Sta = (3S,4S)-4-amino-3-hydroxyl-6-methyl-heptanoic acid, can be manufactured to a very high level of yield and purity (**See the following two graphs on the right**), as well as activity.

Leupeptin (hemisulphate monohydrate MW: 542.7d, CAS: 103476-89-7) is a competitive and reversible tripeptide aldehyde inhibitor of certain serine(trypsin, plasmin, porcine kallikrein), cysteine (papain, cathepsin B) and aspartic proteases. It was also first was isolated from *Actinotomyces.*[2] The synthetic analogue, Acetyl-Leucyl-leucyl-arginal, can also be manufactured to a high level of yield, purity and activity.





Proteins manufactured by intra-cellular mechanisms are subsequently transported intra- and extra-cellularly to perform functions throughout organic systems. Over time, these proteins are irreversibly broken down by endogenous clipping enzymes called proteases (or proteinases). These create protein fragments and shorter peptides that often have important biological functions before being further broken down into constituent fragments and eventually amino acids.

Peptides

During manufacturing by fermentation and microbial expression, therapeutic proteins also are subject to proteolytic degradation, which can reduce process yield and increase cost. These destructive catalytic proteases are self-restoring and remain active in solution until specifically inhibited. Adding protease inhibitors will decrease protealysis, but the inhibitor will also remain in solution until removed by separation tools, such as RP-HPLC.

Catalytic protease inhibitors, natural and synthetic, inhibit the activity of proteases in complex solutions and are used either singly or in cocktails to preserve the integrity and activity of proteins of interest. Over 100 naturally occurring protease inhibitors have been isolated and identified from plants, bacteria and animals.

A significant number of relatively short (<50 residues) inhibitors and analogues have been developed of synthetic, bacterial and fungal origin. Analogues of these short sequences have the potential to be made synthetically.

Proteolytic enzyme activity can be described in two general categories: targeted protealysis, which cleaves a single or a limited number of peptide bonds in a target protein, leading to shorter fragments with increasing specificity of activity; and, progressive protealysis, whereby proteins are degraded into amino acid constituents through various pathways including ubiquitin conjugation and lysosome compartmentation.

Proteases have at least four distinct catalytic mechanisms recognised by the International Union of Biochemistry and Molecular Biology.

The serine proteinases, chymotripsin and substilism families (including bacterial enzymes), all have the essential catalytic triad His^{57}, Asp^{102} and Ser^{195} (chymotripsin nomenclature). Catalysis initiates with the formation of a covalent acyl enzyme intermediate between the substrate and the Ser^{195}, followed by a negatively charged tetrahedral transition state.

Hydrolysis deacylates the acyl-enzyme intermediate and restores the Ser-hydroxyl of the enzyme. The His^{57} provides a base to accept the OH group of the Ser^{195}. Finally the peptide bond is cleaved and the protealytic enzyme is restored.

Aspartic proteinases, mostly of the pepsin family, including fungal proteases, penicillopepsin, rhizopuspepsin and endothiapepsin, are bi-lobed with the active site between two homologous lobes, each with an essential aspartate residue, in a catalytic diad.

Protonation is simultaneously accomplished by a water molecule donation to the two carboxyl groups of the aspartate diad and a concurrent protonation from the diad to the carbonyl oxygen of the substrate resulting in CO-NH cleavage and the protealytic enzyme is restored.

Cystein proteinases, including plant and several mammalian lysosomal cathepsins and some parasitic proteases, have essential Cys^{25} and His^{159} (Papain nomenclature). Metallo proteinases often have a catalylically active zinc atom bound by two essential histidines and one glutamic acid.

Significant advantages of synthetic over biological sources for peptide protease inhibitors include a cleaner impurity profile, with fewer unknown contaminants. Common techniques, such as RP-HPLC, can be used to remove all synthetic elements completely from the protein of interest (many protease inhibitors are APIs[3] and must be completely removed from the pharmaceutical protein product).

Modern raw materials used to manufacture synthetic peptides, namely amino acids, now are commonly synthetically derived and manufactured. Therefore, all putative related impurities in a synthetic peptide, including those below the level of detection using standard assays, are combinations of elements from a known universe of relatively benign raw materials.

For peptides within a defined length, synthetics can be cost-effectively manufactured under cGMP at gram to kilogram scales at significantly lower costs compared to microbial and fermentation methods. Finally, when reduced to lyophilised solids, Pepstatin A and Leupeptin are stable for very long periods when stored at -20˚C.

References:

[1] H. Umezawa, T. Aoyagi, H. Morishima, M. Matsuzaki & M. Hamada, Pepstatin, a New Pepsin Inhibitor Produced by Actinomycetes, *J Antibiot.* (Tokyo) **1970**, *23*, 259-62

[2] T. Aoyagi, T. Takeguchi, M. Matsuzaki, K. Kawamura, S. Kondo, M. Hamada, K. Maeda & H. Umezawa, Leupeptins, New Protease Inhibitors from Actinomycetes, *J Antibiot.* (Tokyo) **1969**, *22*, 283-86

[3] Merck Index *13*, 5477

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Bodipy

BSA/KLH Conjugation

Coumarin

Cy3 and Cy5

Dabcyl

Dansyl

Cyclic peptide through disulfide bridges

Cyclic peptides

EDANS

Farnesyl

FITC

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Palmytoyl

PEGylation

Phosphorylation

PNA (para-Nitroaniline)

Rhodamine 110

Rhodamine B

Ser (octanoic acid)

Stearic acid modification

TAMRA (Tetramethylrhodamine)

Texas Red

Sulfated peptide [Tyr(SO3)]

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