Peptides: Impact of new technologies on chemical production

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INTRODUCTION

The value of peptides as therapeutics has been recognised for a long time and their properties, which are easy to fine-tune, fit in the trend towards a highly potent and selective medication. For biopolymers like peptides, evolution has already realised a discrimination for highly specific and potent compounds. In this learning process, undesired properties like side effects and toxicity have already been eliminated, since peptides had to be adapted to fulfil a given task with utmost precision. Their history and the importance for biological systems suggest an enormous potential for peptides and proteins as drugs. In the past, for pharmaceutical companies, peptides were regarded as a niche market and, consequently, this class of molecules had not been considered for development. In recent years, this attitude has changed and a wealth of information, generated from various disciplines including the proteomics environment, led to a revival of peptides at big pharma (Figure 1).
This trend is supported by recent approvals for peptide drugs like Byetta, Fuzeon, Natrecor, Teriparatide and Symlin which demonstrate the utility of peptides for broad indications like bone metabolism disorders, cardiovascular diseases, type II diabetes and viral infections. In addition, the growing interest in therapeutic antibodies and proteins further endorsed the interest in peptides, since peptides can be viewed as replacement for more complicated structures. Hematide, a synthetic peptide-PEG conjugate developed by Affymetrix, which is now in phase II clinical trials for the treatment of anaemia, represents a prominent example for this strategy. In terms of half life, this peptide compared favourably well with the 2nd generation protein drug Aranesp of Amgen.

The enhanced interest in peptides and the growing market for peptide therapeutics naturally translated into a demand for production of the corresponding APIs. Consequently, technologies relevant for production and characterisation have gained increased attention. These technologies have a considerable impact on production, especially in view of the ever increasing demand for purity and the need for synthesis of more complex structures. As a consequence, there is a necessity for the manufacturer to steadily implement new technologies to meet these ambitious demands. Of course, for API production, issues like trust, regulatory compliance, customer orientation and the proximity to important clients are key to success for a CMO active in the business. In this article, another important aspect, the recent technology developments, in view of the increasing demands from the customer side, are summarized. The perspective of a peptide manufacturer is taken to highlight the significance of new methods for the

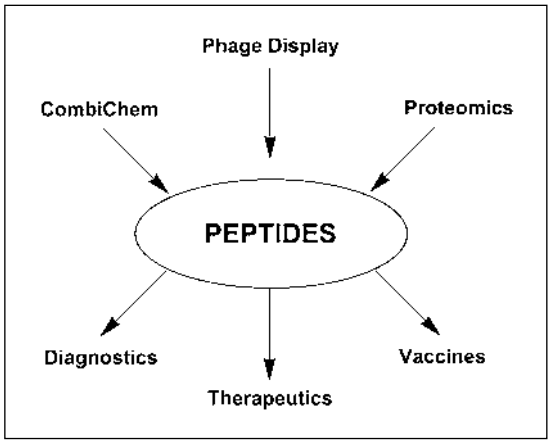

Figure 1 – Peptides: Activities supported by new technologies

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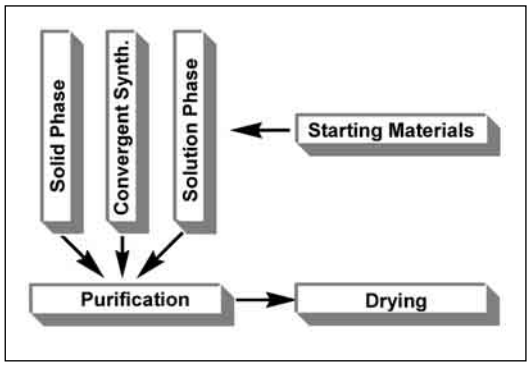

Figure 2 – The production process

production process, albeit, these technologies are of relevance for the production of other APIs. The ability to readily implement new methods will be key to success due to rising pressure to increase efficacy. As a consequence, innovative process development will be mandatory to keep ahead of the growing competition in the peptide manufacturing business.

HISTORY OF PEPTIDE MANUFACTURING

Due to long standing history in peptide chemistry and the availability of appropriate analytical methods, manufacturing of peptides has improved to an extraordinary level. Special techniques have been introduced for synthesis of a variety of peptide derivatives and automation has evolved to address production of hundreds of peptides in a parallel fashion. This continuous methodological fine-tuning, supported by elaborated adjustment of conditions for synthesis, by further development of chromatographic methods and advances in analytical procedures, enables professional peptide chemists to address the synthesis including the proof of homogeneity for even large peptides up to proteins. Nowadays, even smaller proteins are amenable to stepwise chemical synthesis under cGMP conditions. This trend towards more difficult structures requires the work of highly skilled chemists and technicians to implement progress in technologies in the manufacturing process. These advancements include the evolution of the corresponding instrumentation to aid in large scale peptide manufacturing which aids to arrive at more efficient and robust processes. However, in this contribution, the focus resides on recent technologies devised to come up with solutions that address problems from the chemistry side. It is anticipated that these methodologies, as outlined below, will find more widespread application for production including the GMP environment.

Figure 2 provides a comprehensive picture of the production process for chemical peptide fabrication. The first step represents the manufacturing of starting materials or the proper choice for in-sourcing, in particular the protected amino acids. Enhanced purity of these derivatives is inherently correlated to an improved quality of the final API. Thus, in the case of outsourcing, the selection of reliable vendors for high quality derivatives is of prime importance, especially if the synthesis of longer peptides is targeted. In the next step, the chemical production and, most importantly, the customized process is of relevance. Methodological improvements, summarized in this overview, concentrate on this part of the process, in particular on the underlying chemistries, which have been developed to address various problems observed in peptide synthesis.

For peptide manufacturing, traditionally solution phase methods and later solid phase technology, originally described by R.B. Merrifield who received the Nobel prize for his invention in 1984 (1), have been applied. In some cases, a hybrid technology, the convergent synthesis offers advantages for the manufacturing of larger molecules, if e.g. peptide fragments are assembled on the solid support.

Solution phase methods have recently been developed further to enable a faster and more cost effective process, however the option for crystallization of the intermediate had to be sacrificed. Consequently, application of a process based on successive extraction only, may not find general application. On the other hand, no significant advances in protecting group strategies have been reported, although there is a need for more hydrophilic protection to prevent aggregation and to facilitate purification.

Although the solid phase process is more robust, faster, and easier to transfer, in depth knowledge of peptide chemistry is required if problems during synthesis occur. Savings during the development of the synthesis must be carefully compared to the increased efforts needed to establish an effective purification program utilizing expensive chromatographic equipment. Generally speaking, the shorter the peptide and the higher the quantities the more likely a solution phase process is implemented. Since the "limits" have been shifted considerably towards solid phase chemistry, the majority of new projects involve solid phase synthesis procedures, particularly, if structures are complex and time is limited. In addition, solid phase chemistry has become more favourable due to the incremental improvements in coupling protocols, the availability of new resins and the progress in the design of linker/protecting groups with favourable properties. All these efforts led to significant improvements compared to the original Merrifield method. In addition, on the hardware side, modified Rosenmund filter dryers have now been applied for large-scale solid phase synthesis to enable production at the ton level.

For the purification and the drying step several options are available. In addition, the corresponding analytical tools for more advanced quality control have emerged, however, these technologies will not be further discussed in the context of this contribution.

NEW TECHNOLOGIES

The progress in chemistry is not limited to a particular production strategy. Thus, the topics selected for presentation are thought to find more general application and, consequently, are of relevance for different methods available for peptide manufacturing.

Unfortunately, two of the main old headaches of peptide synthesis, aspartimide formation and racemization have not been eradicated. Some progress in optimisation of cleavage protocols and the design of alternative protecting groups for Asp have led to the reduction of the undesired reaction to the aspartimide and subsequent modifications. In the case of the critical Asp-Gly sequence, the coupling of the corresponding backbone protected dipeptide represents a close to optimal solution for this problem (2) (Figure 3). Nevertheless, this approach has not found more general application, due to the difficulties to gain access to other dipeptides and the fact that the backbone modification does not prevent racemization for other amino acids but Gly.

Similarly, coupling reagents and protocols, in particular for critical amino acids like Cys and His, have been customized to keep levels of racemization low. Besides the difficulties in the manipulation of specific building blocks, problems related to secondary structure

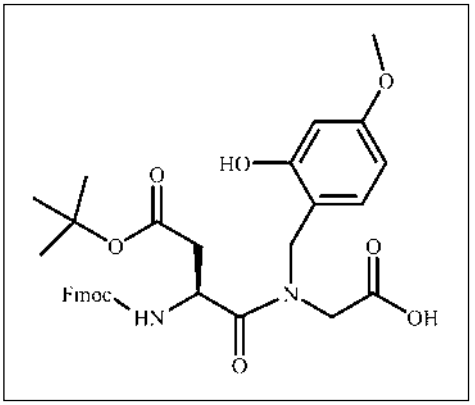

Figure 3 – The derivative Fmoc-Asp(OtBu)-(Hmb)-Gly-OH

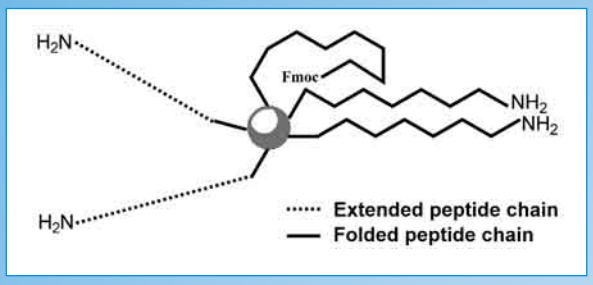

Figure 4 – Peptide chain aggregation for a peptide on a single resin bead

formation and aggregation sometimes raise production costs to the limit or even lead to abandonment of the project. Often, for these so called "difficult" peptides, a decline in coupling yield or incomplete protecting group removal is observed more like an abrupt change following the incorporation of one particular amino acid derivative. As illustrated for the case of solid phase synthesis (Figure 4), partial folding of the growing peptide can either limit accessibility of the temporary protecting group for cleavage (in this case Fmoc) and/or result in steric crowd at N-terminal, preventing complete acylation by the activated amino acid derivative to be coupled. Needless to say0 that in this case, the quality of the crude peptide and, consequently the purity of the final product, is severely compromised. These phenomena are also observed in solution phase synthesis and a perfect solution may turn into a gel upon coupling of a particular building block or a fragment if the critical chain length for aggregation/secondary structure formation has been accomplished. Most of the time, at this point, it is too late for corrective measures and trouble-shooting starts.

The choice of reaction condition, e.g. temperature and/or solvent, offers some room for overcoming these effects and may be helpful to limit the undesired behaviour. In the case of solid phase synthesis, in addition to the options indicated above, the reduction of the resin loading and/or the choice of a support with improved swelling properties may alleviate the problem. However, the breaking of H-bonding patterns, e.g. by innovative protecting group strategies or introduction of a charged group to the molecule, is believed to be an alternative, more generic solution which can be applied in a prophylactic manner. In addition to its beneficial effects for incorporating an Asp-Gly motif in a peptide sequence, the Hmb protecting group, located on the nitrogen of Gly (Figure 3), has some positive effects on sequences with a tendency to aggregate. Due to the lack of a H-donor as compared to the native amide backbone and the presence of a bulky protecting group, this dipeptide building block restricts aggregation of the surrounding peptide chain.

Another possibility to inhibit H-bonding has been introduced for peptides containing Ser and Thr residues (Figure 5), however due to stability problems, these so called pseudo prolines have also to be coupled as the corresponding dipeptide building blocks (3). Especially the constraints of the cyclic structure and their impact on neighbouring sequence elements leads to suppression of β-sheet formation and aggregation.

As a consequence, the application of pseudo prolines can have a tremendously positive effect on the purity of the crude peptide. For longer sequences, a repetitive incorporation of an adequate building block is required if the segment has a tendency to aggregate and, of course, the necessity for the presence of Ser or Thr residues in the peptide sequence represents a limitation of this promising strategy. Interestingly, under standard conditions applied for carboxy activation, pseudo prolines do not racemize. Thus, besides

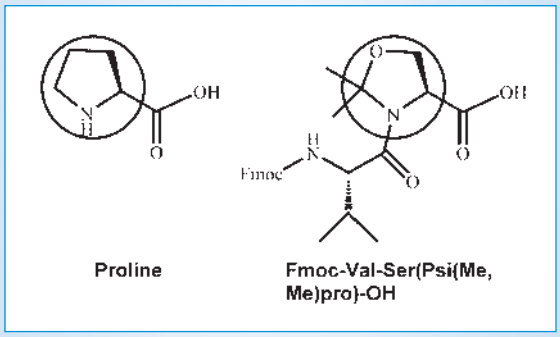

Figure 5 – Structure of a pseudo-Pro derivative as compared to Pro

their beneficial effects on peptide aggregation, pseudo prolines enhance the scope of classical fragment condensation strategies which, due to the racemization problem, are usually restricted to the use of Gly- and Pro-sites.

Charge repulsion represents an alternative to disable H-bonding and can also help to overcome problems related to the aggregation of peptide chains. Earlier work by Kent has demonstrated the advantage of *in situ* neutralization for Boc assisted synthesis (4). In this case, the N-terminal of the protected peptide carries a charge, which may be present as the result of protecting group cleavage. The protonated amino function is only neutralized upon addition of the coupling cocktail which contains a stoichiometric amount of base to liberate the amino group. Accordingly, Coulomb forces keep the peptide chains segregated until the coupling cocktail neutralizes the charge and immediate acylation leads to peptide bond formation before congregation can occur. In a recent publication, *in situ* neutralization has been successfully applied to improve the synthesis of the peptide CGRP (8-37) (5).

Another method to prevent aggregation by disrupting the peptide backbone structure uses isopeptide bonds (Figure 6). Here, an ester bond to a Ser side chain leads to interruption of the regular backbone amide structure and, as a consequence, secondary structure formation is aggravated. In this particular case, following assembly of the molecule, the charged amino function is generated only post assembly, in the course of acid catalysed protecting group cleavage. Since purification is usually carried out under acidic conditions, the isopeptide bond remains intact and the charged amino functions can facilitate the purification process. Upon liberation of the free amino group, the isopeptide is then easily converted to the native peptide backbone. Based on this principal, a process for the sparingly soluble human Amyloid β (1-42) peptide has been designed (6).

Although this method has considerable beneficial effects for synthesis and purification, the limited stability of the ester group, e.g. under the basic Fmoc cleavage conditions, represents a critical issue and may limit the synthesis of long peptides.

Recently, an ionic liquid type protecting group has been introduced with the aim to expand the scope of solution phase peptide synthesis (7), albeit results have been presented only for a penta-peptide. The ionic liquid tag permits selective extraction steps and offers opportunities for purification of the intermediate. The permanent charge on the imidazole moiety of the ionic liquid component serves the purposes as discussed above and the idea to investigate more hydrophilic protecting groups seems worthwhile to overcome problems related to aggregation phenomena. On the other hand, a single ionic liquid moiety will not be sufficient to support this alternative solution phase process for slightly longer peptides due to decreasing effect of the charge on the overall properties of a larger molecule.

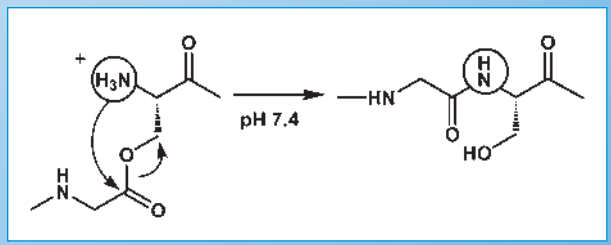

Figure 6 – Principal mechanism for converting an isopeptide to the natural backbone structure

Especially, the on-going technology development with the aim to influence secondary structure formation/aggregation during production and purification will permit a more reliable synthesis of larger and more complex peptides up to proteins in the future.

Historically, the production of larger amounts of peptides has been accomplished by solution phase methods, since this process provides options for purification of the intermediates and scale-up. However, for the production of larger molecules, the efficacy of solution synthesis is limited, and as outlined above, the simplicity of a solid phase approach and the speed represent the competitive advantages for this method. As a result, solid phase peptide synthesis will be the method of choice for the synthesis of larger peptides and/or proteins. Steady improvements in the various chemistries have led to considerable overall progress and even small proteins like chemokines are accessible in good quality by a stepwise solid phase approach. Although premature with respect to large scale production, alternative, chemical methods for protein synthesis are summarized to conclude this overview on technology developments important for peptide manufacturing.

The limitations of stepwise synthesis with respect to the production of longer peptides and proteins have already been overcome following introduction of the native chemical ligation method described by Kent in 1994 (8). Proteins containing well over 100 amino acids have been synthesized and subsequent purification including correct folding has been realized. This technology is based on the unique reaction of a fragment carrying an N-terminal Cys with a 2nd fragment moderately activated by a thioester at the C-terminal (Figure 7, Kent). Both fragments need no protecting groups and the ligation occurs under aqueous conditions at neutral pH. Unfortunately, the use of the reaction is somewhat restricted, since the system requires the presence of a Cys residue which is one of the more rare amino acids found in natural peptides and proteins. To overcome this limitation, thiol-containing backbone protecting groups have been designed. Unfortunately, they proved only helpful for sequence motifs containing simple amino acids like Gly and did not achieve the efficacy as the original Cys-ligation reaction. Nevertheless, native chemical ligation has opened up the doors for chemical protein synthesis and even methods have been worked out to enable sequential, racemization-free coupling of peptide fragments on the basis of this ligation technology.

In the beginning of 2006, another chemo-selective reaction has been introduced. In this case, the native peptide backbone is generated by an unusual decarboxylative condensation if a hydroxylamine derivative is reacted with an α-ketoacid (Figure 7, Bode) (9). This method has the potential to overcome sequence-related limitations of the thioester mediated reaction, however its benefits for protein synthesis and the broad applicably still remain to be established.

Expressed protein ligation, based on the Cys ligation originally described by Kent, offers opportunities to combine biotechnology and chemistry (10). The expression system employed is able to provide large protein molecules with a C-terminal thioester moiety that can be used to couple the recombinant protein portion to a Cys-containing fragment produced by solution or solid phase peptide synthesis. This method for semi-synthetic protein production permits the site specific incorporation of non-natural modifications into a protein.

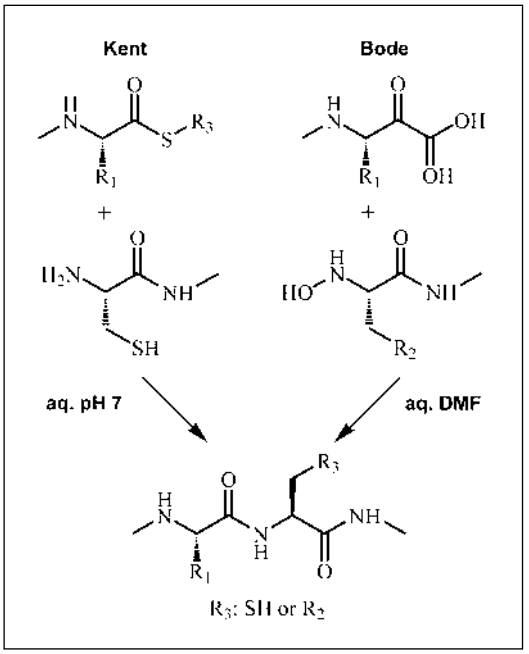

Figure 7 – Ligation reactions according to Kent (8) and Bode (9)

Clearly, protein production by chemical methods has become feasible and access by synthetic methods will continuously improve. Interestingly, the requirements on the commercial level for highly selective and potent therapeutic peptides or proteins are in the low kg-range. Thus, a synthetic process may be competitive with recombinant production, especially, if site specific modifications, either corresponding to natural posttranslational modification or to alterations to modify half-life, have to be introduced. As a consequence, the development of synthetic protein therapeutics may be an option for biotech/pharma industries to be able to finetune their properties. Looking ahead, manufacturers have to be prepared for production of peptides/proteins exhibiting another level of complexity to be able to succeed in supply at larger scale.

CONCLUSIONS

In summary, technologies for production of peptides have been and will be further developed to satisfy the growing need for expert synthesis of new molecules. In addition, the interest of Biotech/Pharma industries to replace monoclonal antibodies and proteins by simpler structures with the same efficacy will support this uphill-trend of peptides as therapeutics. As a consequence, manufacturers will have to implement innovative processes and up-to-date technical equipment to be able to the meet market demand and, thus, to remain competitive in this dynamic environment. The potential for some of the peptides, either in development or already on the market, to achieve blockbuster status will also encourage other API manufacturers to invest in peptide technology. However, besides other reasons, the unique properties of peptides and the problems associated with their production represent an efficient entry barrier for non-experienced fine chemical companies targeting this market. As a result, companies already active in the production of peptides are in a comfortable position, especially if they can keep up with the latest developments and readily implement recent technologies in the production process to ensure state-of–the-art cGMP-supply at a reasonable price.

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