Protein Hydrolysates in Biotechnology

Vijai K. Pasupuleti · Arnold L. Demain
Editors

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Springer

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ISBN 978-1-4020-6673-3 e-ISBN 978-1-4020-6674-0
DOI 10.1007/978-1-4020-6674-0
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2010934419

Preface

Protein hydrolysates, otherwise commonly known as peptones or peptides, are used in a wide variety of products in fermentation and biotechnology industries. The term "peptone" was first introduced in 1880 by Nagelli for growing bacterial cultures. However, later it was discovered that peptones derived from the partial digestion of proteins would furnish organic nitrogen in readily available form. Ever since, peptones, which are commonly known as protein hydrolysates, have been used not only for growth of microbial cultures, but also as nitrogen source in commercial fermentations using animal cells and recombinant microorganisms for the production of value added products such as therapeutic proteins, hormones, vaccines, etc.

Today, the characterization, screening and manufacturing of protein hydrolysates has become more sophisticated, with the introduction of reliable analytical instrumentation, high throughput screening techniques coupled with statistical design approaches, novel enzymes and efficient downstream processing equipment. This has enabled the introduction of custom-built products for specialized applications in diverse fields of fermentation and biotechnology, such as the following.

1. Protein hydrolysates are used as much more than a simple nitrogen source. For example, the productivities of several therapeutic drugs made by animal cells and recombinant microorganisms have been markedly increased by use of protein hydrolysates. This is extremely important when capacities are limited.
2. Protein hydrolysates are employed in the manufacturing of vaccines by fermentation processes and also used as vaccine stabilizers.
3. Protein hydrolysates are being used in large-scale industrial fermentations as sources of nitrogen and unknown growth factors, such as certain peptides, etc. They are also useful in diagnostic media to grow microorganisms in Petri plates and to detect pathogens and perform antibiotic sensitivity tests.
4. Protein hydrolysates are used in regular diets as well as prescription diets for companion animals.
5. Protein hydrolysates play an important role in animal nutrition, especially for raising healthy animals with increased immune resistance.
6. Protein hydrolysates are used as plant growth regulators to increase commercial crop yields as well as to control weeds in plants.

Thus protein hydrolysates industry is growing rapidly with wide applications in biotechnology, yet there has been no single book that describes (i) the challenges and opportunities for manufacturers and end users, (ii) the techniques used in manufacturing, characterization and screening of protein hydrolysates, and (iii) the applications of protein hydrolysates in a wide variety of industries, e.g., that of fermentation for production of many primary and secondary metabolites of microorganisms, and in the rapidly growing area of industrial biotechnology for production of biopharmaceuticals. One of the misconceptions involving the use of protein hydrolysates in fermentations is that they are being used merely as a nitrogen source. However, the functionality of the product obtained is not necessarily due solely to protein hydrolysates in general, i.e., it may be due to specific peptides, or the combination of peptides, or to non-protein components in the product. This is due to the fact that the preparations may contain carbohydrates, lipids, micronutrients, etc. Indeed, some manufacturers deliberately add such factors into the process to bring about unique functionality. Since only a handful of manufacturers dominate the market, this tends to keep the manufacturing process proprietary, making it harder to understand and appreciate its fine points. This book will close the gap by revealing valuable information on the latest developments in this vital and tremendously important field.

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Acknowledgements

Our special thanks are due to all the contributors; without their dedication, hard work, patience and time to share their expertise; this book would not have been possible.

We also thank Jacco Flipsen for coordinating the book and Ineke Ravesloot for editorial assistance from Springer publishers.

VKP likes to thank his father P.V. Subba Rao; wife Anita; and sons, Anoop and Ajai, for providing support, energy and enthusiasm in completing this book.

ALD appreciates the patience of his wife JoAnna for all those hours he spent on the computer and his daughter, Pamela, son, Jeffrey, and colleague Vince Gullo for their assistance in solving computer problems. He also expresses thanks to coauthor Vijai for inviting him to participate in the editing of this fine book.

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Chapter 1
Applications of Protein Hydrolysates in Biotechnology

Vijai K. Pasupuleti, Chris Holmes, and Arnold L. Demain

Abstract By definition, protein hydrolysates are the products that are obtained after the hydrolysis of proteins and this can be achieved by enzymes, acid or alkali. This broad definition encompasses all the products of protein hydrolysis – peptides, amino acids and minerals present in the protein and acid/alkali used to adjust pH (Pasupuleti 2006). Protein hydrolysates contain variable side chains depending on the enzymes used. These side chains could be carboxyl, amino, imidazole, sulfhydryl, etc. and they may exert specific physiological roles in animal, microbial, insect and plant cells. This introductory chapter reviews the applications of protein hydrolysates in biotechnology. The word biotechnology is so broad and for the purpose of this book, we define it as a set of technologies such as cell culture technology, bioprocessing technology that includes fermentations, genetic engineering technology, microbiology, and so on. This chapter provides introduction and leads to other chapters on manufacturing and applications of protein hydrolysates in biotechnology.

Keywords Protein hydrolysates • Biotechnology • Applications • Cell culture

Introduction

Protein hydrolysates, commonly known as peptones or peptides, are used in a wide variety of products in the fermentation and biotechnology industries (Pasupuleti 2006). The art of manufacturing and application of protein hydrolysates goes back

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V.K. Pasupuleti and A.L. Demain (eds.), *Protein Hydrolysates in Biotechnology*,
DOI 10.1007/978-1-4020-6674-0_1,

to the days when meat hydrolysates began their use to grow microbial cells (Nagelli in general, i 1880). Today, the manufacturing of protein hydrolysates has become more sophisticated with the introduction of novel enzymes and efficient downstream processing equipment to custom-build products for specialized applications in biotechnology (Heidemann et al. 2000).

With the increased knowledge, understanding and sophistication of manufacturing of protein hydrolysates has led to their applications in areas of fermentation and biotechnology such as medicine, agriculture, industrial fermentations, production of recombinant proteins, diagnostic media, bioremediation, weed control for plants and for healthy growth of young animals and companion animals. Throughout this book, authors have discussed these specialized applications of protein hydrolysates.

Protein hydrolysates are particularly useful in the biotechnological production of monoclonal antibodies (mabs), peptides and therapeutic proteins.

1. The contribution of protein hydrolysates is much more than that of supplying simple nitrogen sources. They are useful in increasing monoclonal antibody production by some unknown mechanism, and also for increasing the productivities of several therapeutic drugs made by animal cells and recombinant microorganisms. Chu and Robinson (2001) have reviewed the FDA biological license approvals from 1996 to 2000 and found that 21 out of 33 were manufactured using mammalian cell culture. The media of one third of these have not been revealed and only one company claimed to use protein hydrolysates in the medium. Anecdotal evidence suggests that more manufacturers use protein hydrolysates in their media and keep it as a trade secret. Some manufacturers file the patents and a partial list of patents is given in the reference section (World Patents). A sampling of published US Patents/Applications by major biopharmaceutical companies using protein hydrolysates by a variety of cell lines is discussed in Chapter 3.

   Protein hydrolysates are also used in the manufacture of vaccines and as an adjuvant in vaccines as seen in Table 1.

   Chapter 2 reviews in detail with the state of the art of manufacturing of protein hydrolysates for applications in biotechnology. The role of protein hydrolysates, their benefits and limitations in animal cell culture are covered in detail in Chapters 3–5.
2. Protein hydrolysates are widely used in the manufacture of probiotics, starter cultures and fermented products. Sobharani and Agrawal (2009) have demonstrated that the viability of probiotic culture was enhanced with supplementation of adjuvants like tryptone, casein hydrolysate, cysteine hydrochloride and ascorbic acid. Chapter 6 reviews the use of protein hydrolysates in industrial starter culture fermentations.
3. In industrial fermentations, protein hydrolysates have been used for several decades to supply nitrogenous compounds such as peptides, amino acids and to increase productivities and yields. They are also employed in diagnostic media to grow micro-organisms (BD Bionutrients Technical Manual 2006). Chapter 7 reviews replacement of tryptone in microbiological media by protein hydrolysates from non-bovine and plant sources.

Table 1 Excipients included in US vaccines. Includes vaccine ingredients (e.g., adjuvants and preservatives, protein hydrolysates are highlighted) as well as substances used during the manufacturing process, including vaccine-production media that are removed from the final product and present only in trace quantities

Vaccine	Contains
DTaP (Daptacel)	Aluminum phosphate, ammonium sulfate, **casamino acid**, dimethyl-betacyclodextrin, formaldehyde or formalin, glutaraldehyde, 2-phenoxyethanol
DTaP-IPV (Kinrix)	Aluminum hydroxide, bovine extract, formaldehyde, **lactalbumin hydrolysate**, monkey kidney tissue, neomycin sulfate, polymyxin B, polysorbate 80
DTaP-HepB-IPV (Pediarix)	Aluminum hydroxide, aluminum phosphate, bovine protein, **lactalbumin hydrolysate**, formaldehyde or formalin, glutaraldhyde, monkey kidney tissue, neomycin, 2-phenoxyethanol, polymyxin B, polysorbate 80, yeast protein
Hib/Hep B (Comvax)	Amino acids, aluminum hydroxyphosphate sulfate, dextrose, formaldehyde or formalin, mineral salts, sodium borate, **soy peptone**, yeast protein
Hep B (Recombivax)	Aluminum hydroxyphosphate sulfate, amino acids, dextrose, formaldehyde or formalin, mineral salts, potassium aluminum sulfate, **soy peptone**, yeast protein
Pneumococcal (Prevnar)	Aluminum phosphate, amino acid, **soy peptone**, yeast extract
Zoster (Zostavax)	Bovine calf serum, **hydrolyzed porcine gelatin**, monosodium L-glutamate, MRC-5 DNA and cellular protein, neomycin, potassium phosphate monobasic, potassium chloride, sodium phosphate dibasic, sucrose

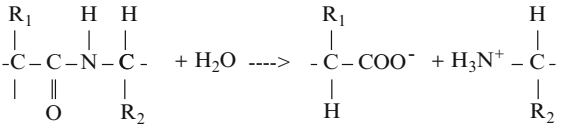
Downloaded on June 16, 2009 from http://www.cdc.gov/vaccines/pubs/pinkbook/downloads/appendices/B/excipient-table-2.pdf

4. Special applications of protein hydrolysates include use as plant growth regulators and to increase pest resistance to plants (Inagrosa 2002; Figueroa et al. 2008). Protein hydrolysates are used in bioremediation, especially to boost the growth of microorganisms. Chapter 8 reviews the use of protein hydrolysates for weed control.
5. Chapter 9 provides a detailed review of the physiological importance and mechanisms of protein hydrolysate absorption. For animal and pet nutrition, protein hydrolysate are being used in regular as well as prescription diets of companion animals. Chapters 10 and 11 review in detail the applications of protein hydrolysates in animal nutrition and companion animals.

Finally, extending the theme of non-animal protein hydrolysates, Chapter 12 discusses the development of novel recombinant human gelatins as replacements for animal-derived gelatin hydrolysates in pharmaceutical applications.

What Are Protein Hydrolysates?

By definition, protein hydrolysates are the products obtained after the hydrolysis of proteins as achieved by acid, alkali, enzymes and fermentation methods. This broad definition encompasses all of the products of protein hydrolysis, i.e., peptides,



Adapted from Jens Adler-Nissen (1986)

amino acids, minerals, carbohydrates and lipids if they are present in the substrate or enzyme (sometimes animal tissues are used as an enzyme source) along with the protein.

The protein hydrolysates are typically characterized by the degree of hydrolysis, i.e., the extent to which the protein is hydrolyzed. The degree of hydrolysis (DH) is measured by the number of peptide bonds cleaved, divided by the total number of peptide bonds and multiplied by 100. The number of peptide bonds cleaved is measured by different test methods and the most popular are: Formol titration (Sorensen, 1907) or the OPA method (Church et al., 1985) or the trinitrobenzene sulphonic acid, TNBS (Fields 1971) reagent method, and reported as AN (alpha amino nitrogen). The Kjeldahl, or Dumas direct combustion methods, are typically used to measure TN (total nitrogen).

$$\%\mathrm{DH} = \frac{\text{AN of protein hydrolysate} - \text{AN of protein} \times 100}{\text{Total Nitrogen of the protein}}$$

The common practice in industry is to measure AN and TN and multiply by 100 to report the AN/TN ratio which is nothing more than the degree of hydrolysis of the protein.

Protein hydrolysates are commonly known as peptones, protein fission products, peptides and hydrolyzed proteins; (Acid-HP, hydrolyzed acid protein and HVP, hydrolyzed vegetable protein). Typically, Acid-HPs and HVPs are used in the food industries and not in industrial fermentations and animal cell culture because of very high ash and low protein content.

Evolution of Protein Hydrolysates

The first application of protein hydrolysates dates back to 1880 when Nagelli used it to cultivate microbes. The next recorded use was around 1900 for preparation of Voges-Proskauer and McConkey media. In 1914, Difco Laboratories introduced Bacto-Peptone for use in bacteriological media. Since then, the uses of protein hydrolysates have become widely popular for the growth of microorganisms (Fig. 1).

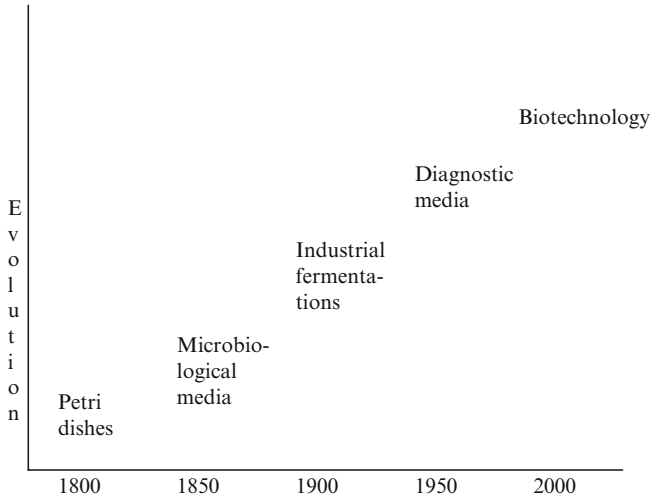


Fig. 1 Applications of protein hydrolysates over the years

Applications of Protein Hydrolysates in the Biotechnology Industry

The word "Biotechnology" is very broad and has been freely and widely used to define a wide variety of topics but here, for the purpose of this book, we define biotechnology in a broader sense that encompasses molecular biology, microbiology, fermentations, animal, insect and plant cell culture, and young animals and pets where protein hydrolysates are used as a nitrogen and nutritional source. Applications of protein hydrolysates in biotechnology are briefly discussed leading to other chapters where they are discussed in greater detail.

1. *Protein production in microbial cells. Escherichia coli* is the most popular bacterium for production of recombinant proteins. The traditional media for this fermentation process contain tryptone (Franchon et al. 2006). With respect to yeasts, *Pichia pastoris* is widely used and its media contain peptone which not only supplies nitrogen, but also reduces protein degradation by acting as an excess substrate for harmful proteases (Cregg et al. 1993). Another yeast often used is *Saccharomyces cerevisiae* and, in this case, peptone is also the usual nitrogen source (Penttila et al. 1987). With filamentous fungi such as *Aspergillus awamori*, again peptone is the favored nitrogen source (Lamsa and Bloebaum 1990).
2. *Protein production in mammalian cells*. Production of proteins, including monoclonal antibodies, in mammalian cells is usually carried out in Chinese Hamster Ovary (CHO) cells or non-secreting mouse myeloma (NSO) cells using

media containing animal serum. However, it is interesting to note that as early as Taylor et al. (1972 and 1974) studied a serum substitute for mammalian cells in culture using peptones. Until the Bovine Spongiform Encephalopathy (BSE) issue have became serious, serum was the favorite but after that, the trend has shifted towards serum-free media. This situation in which humanized antibodies are now produced in fungal cells such as *P. pastoris* (Kunert et al. 2008) or *Aspergillus niger* (Ward et al. 2004). This trend could see expanded use of protein hydrolysates, especially those from vegetable proteins like soy, wheat and pea in the future.

3. *Protein production in insect cell cultures.* The baculovirus expression vector system is the common process for producing proteins using insect cells. Such a system using *Spodoptera frugiperda* (Sf-9) cells employs a medium that contains lactalbumin hydrolysate (Neutra et al. 1992). Most insect cell media contain fetal bovine serum which is the most expensive component of the medium, is variable from lot-to-lot, may contain cytotoxic materials, is susceptible to contamination by viruses and mycoplasma, and causes problems such as foaming and difficult product purification. Furthermore, serum is not needed for insect cell growth and much effort has been put into serum-free media which often contain peptone or lactalbumin hydrolysate (Agathos et al. 1990).
4. *Vaccines.* The use of bovine serum to make certain vaccines has been looked down upon because of a possible adverse effect of prion diseases such as Mad Cow Disease (BSE). In such a disease, prions infect neurons of the brain. Thus, vegetable protein hydrolysates can be very useful for replacement of animal protein hydrolysates in preparation of media for production of toxins to be used to produce animal-free vaccines. For example, in the preparation of tetanus toxin, which is used to make the toxoid for immunization, growth of *Clostridium tetani* traditionally is done in media containing animal and dairy products (e.g., meat extracts, brain heart infusion, casein hydrolysates). As a result, toxoids often contain undesirable formalin adducts of animal proteins. To avoid this problem, a new medium containing hydrolyzed soy proteins has been devised which yields even higher titers of tetanus toxin than the old traditional medium (Demain et al. 2005, 2007; Fang et al. 2006).
5. *Plant cell culture.* The most important product made in plant cell culture is Taxol (paclitaxel), one of the most successful anti-cancer agents known. It is produced in plant cell suspension culture by *Taxus chinensis* and *Taxus yunnanensis*. The standard medium for the former culture contains casein hydrolysate (Choi et al. 2000) while that of the latter includes lactalbumin hydrolysate (Zhang et al. 2002).
6. *Production of primary metabolites.* One of the major contributions of microorganisms in industry is the production of primary metabolites such as amino acids, vitamins, flavor nucleotides, etc. Many of these fermentation processes utilize protein hydrolysates. For example, manufacture of L-glutamic acid is the major primary metabolite made in industry (1.5 million tons per year). *Corynebacterium glutamicum* is one of the cultures used to produce glutamic acid commercially in a medium containing soybean protein hydrolysate (Kataoka et al. 2006).

Future Directions

The most important development that has to take place is the partnership between protein hydrolysate manufacturers and the end users. This partnership will enable understanding of the capabilities of manufacturers and the requirements of end users which may lead to more defined and better products.

Genetic engineering might play a role in developing animal-free and defined products. One of the recent developments is to produce protein hydrolysates (peptides) by fermentation. Olsen et al. (2010) describes the whole process in detail in Chapter 12.

Major companies like Ajinomoto, Kyowa Hakko and others are manufacturing peptides by fermentation for use in cell culture and industrial fermentations. We hope that in the future, more products and processes like this will come into play as they not only alleviate the BSE problem but also provide consistency from batch to batch and provide higher confidence levels similar to defined media components.

Sophisticated analytical techniques such as NMR, MALDI/TOF, peptide mapping are being applied to characterize (fingerprint) protein hydrolysates and this coupled with spent media analysis may give better insight. This knowledge could be potentially utilized in the manufacturing of protein hydrolysates to tailor-make products for specific applications and to avoid variations from batch to batch.

Manufacturers, by using standardized units of enzyme, instituting in-process controls utilizing membrane technologies for purification, and establishing functional specifications, will get closer to producing consistent products.

One could foresee a major development in the enzymes used for manufacturing protein hydrolysates.

The design of experiments using factorial or fractional factorial methods and high throughput screening enables the end user to screen a variety of protein hydrolysates and dose response. Perhaps a combination of protein hydrolysates would be better in some instances.

We are optimistic and believe that in the near future all of the above technologies and partnerships between the manufacturers and end-users will bring exciting new developments in the applications of protein hydrolysates in biotechnology.

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Chapter 2
State of the Art Manufacturing of Protein Hydrolysates

Vijai K. Pasupuleti and Steven Braun

Abstract The use of protein hydrolysates in microbiological media has been in existence for several decades and the basic manufacturing process of protein hydrolysates has remained the same. However, with increasing use of protein hydrolysates in specialized applications such as animal cell culture processes, the manufacturing of protein hydrolysates has dramatically improved and is still in its infancy to uncover the specific peptide, peptides and combination of individual amino acids that produce intended effects for that application. This will change as the protein hydrolysate manufacturers and end-users exchange information and work towards the common goal of developing the best protein hydrolysates for specific applications. This chapter will review the generic manufacturing of protein hydrolysates describing individual unit operations, problems faced by manufacturers and suggestions for obtaining consistent product and guidelines for the end-users in getting regulatory support and setting up reliable specifications. Finally the chapter concludes with future trends of protein hydrolysates.

Keywords Manufacturing • Protein hydrolysates • Downstream processing • Inconsistencies • Hydrolysis

Introduction

The most basic function of protein hydrolysates in the applications of biotechnology is to provide a nitrogen source for bacteriological, industrial and specialized media for microbial, plant, animal and insect cell cultures on both a laboratory and industrial scale. However, in many instances protein hydrolysates also provide vitamins,

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DOI 10.1007/978-1-4020-6674-0_2,