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,

V.K. Pasupuleti (✉)
SAI International, Geneva, IL 60134, USA
e-mail: Vijai1436@sbcglobal.net

S. Braun
Mead Johnson Nutrition, Evansville, IN 47721, USA

V.K. Pasupuleti and A.L. Demain (eds.), *Protein Hydrolysates in Biotechnology*,
DOI 10.1007/978-1-4020-6674-0_2,

minerals and unknown growth factors resulting in higher yields and productivities (Pasupuleti and Schie 1998). For more details the reader is encouraged to refer to the Chapters 1, 3–7 for detailed discussions on use of protein hydrolysates with specific reference to industrial fermentations, cell culture and microbiological media.

The current modern manufacturing facilities with sophisticated equipment and technology for manufacturing protein hydrolysates are a result of long standing practice, extensive research and to a certain extent trial and error starting from the first attempt to grow microbial and animal cells. During the early days, the nitrogen requirements of bacteria grown in the laboratory were met by adding naturally occurring substances like blood, body fluids, etc., to the media. The first report on the use of egg albumin was published in 1882 by Naegeli, which he referred to as "peptone," a term still used today for some products. However, later it was discovered that peptones derived from the partial digestion (hydrolysis) of proteins would furnish organic nitrogen in a more available form (Peters and Snell 1953; Pasupuleti 1998). These peptones that are commonly known as protein hydrolysates are now the preferred source of nitrogen for bacteriological culture media, commercial fermentations and specialized cell culture processes. From its first use as an improved source of nitrogen in bacteriological media, protein hydrolysates are now used widely in other areas of biotechnology such as:

- Animal cell culture for the production of monoclonal antibodies, therapeutic proteins, enzymes, etc. (Pasupuleti 1998, 2001; Ganglberger et al. 2005).
- Recombinant culture fermentations for the manufacture of therapeutic drugs, vaccines, etc. (Mazurkova et al. 2008; Kwon et al. 2008; Heidemann et al. 2004; Tripathi et al. 2009).
- Insect and plant cell cultures for a variety of end products (Ikonomou et al. 2001, Kwon et al. 2005; Girón-Calle et al. 2008).
- Specialized media for growing and expressing the genetically modified micro-organisms (Blattner 1977; BD Manual 2009).
- Animal feeds for higher milk output, better meat quality, increased weight gain in shorter time frames (Backwell 1998; Choung and Chabmberlain 1998; Gilbert et al. 2008; Cordoba et al. 2005).
- Crops for enhanced yields and productivities (Kinnersley et al. 2003; El-Naggar 2006).

Many if not all of these newer applications require higher quality specialty protein hydrolysates with stringent specifications rather than the crude preparations that were initially manufactured. To meet this demand, the industry is responding with newer protein hydrolysates that are an excellent nutritional source providing di, tri, oliogpeptides and amino acids, which exert specific physiological functions to increase the productivities of cell culture and fermentations (Pasupuleti 2000). This field is rapidly growing and the sales of protein hydrolysates are increasing year after year and the manufacturers are refining their processes to better define their products. Still to a larger extent especially in the cell culture applications, much of the work is trial and error because complete characterization of protein

hydrolysates with respect to the requirements of animal cells is not as well known as that seen in defined media (Pasupuleti 2007).

In this chapter, we focus mainly on the manufacturing of protein hydrolysates related to biotechnology applications and it is beyond the scope of this chapter to cover the manufacturing of protein hydrolysates for nutraceuticals and food applications. This chapter will review:

1. Current practices of different manufacturing methods of protein hydrolysates: acid, alkali, enzymatic hydrolysis of proteins and briefly about upcoming novel manufacturing of peptides by enzymes and fermentations. A generic flow of manufacturing process of protein hydrolysates will be described.
2. Selection of raw materials, protein solubilization, pretreatment methods, controlling and monitoring of microorganisms during the manufacturing, significance of proteases, the degree of hydrolysis, AN/TN ratio, peptide chain length, molecular weight distribution profiles, free and total amino acids will be highlighted.
3. Downstream processing which is dictated by the end use of protein hydrolysates: reviews brief description of plate and frame filtration and carbon treatment methods. Centrifugation, micro, ultra and nano filtration, reverse osmosis, column chromatography, ion exchange will not be covered in this chapter.
4. Different methods of concentration and drying: spray dried powders; roller drum dried powders and agglomerated powders.
5. Considerations in plant design and layout: importance of the source of water, ISO/GMP facilities.
6. Future trends.

Current Practices of Different Manufacturing Methods of Protein Hydrolysates

The predominant manufacturing method of protein hydrolysates for applications in biotechnology is by enzymes. However, protein hydrolysates made by acid and alkaline hydrolysis of proteins are also commercially available.

Acid hydrolysis of proteins: The first reported acid hydrolysis goes back to 1820 by Braconnot (Hill 1965). It took several decades for commercialization and is still in practice representing one of the older processes. The majority of acid hydrolyzed proteins are used as flavor enhancers and only a small portion of these acid hydrolysates are being used in biotechnology, sometimes after partially or completely removing the salt (Nagodawithana 1995).

Hydrochloric and sulfuric acid are mainly used to hydrolyze proteins, the most common being hydrochloric acid. With the acid hydrolysis some of the essential amino acids such as tryptophan, methionine, cystine and cysteine are destroyed. Further, glutamine and aspargine are converted to glutamic acid and aspartic acid (Bucci and Unlu 2000). Typically, acid hydrolysis breaks down the proteins into individual amino acids and minute amounts of smaller peptides. The process adds significant amounts

of salt, which is detrimental to the growth of microorganisms. For this reason some of the manufacturers remove salt partially or completely by precipitation nanofiltration and/or ion exchange resins. However, acid hydrolysates are widely used in the food and pet food industry as flavor enhancers (Nagodawithana 1998, 2010). This is covered in greater detail in Chapter 11.

The manufacturing of acid hydrolysates requires glass lined stainless steel reactors that can withstand high pressure and temperatures. Equally important are the safety procedures built around these processes. The governing factors for acid hydrolysis are the concentration and type of acid (hydrochloric acid or sulfuric acid), temperature (250–280°F), pressure (32–45 psi), time of hydrolysis (2–8 h) and the concentration of protein (50–65%). All of these independently and combined will have an impact on quality of the product.

Acid hydrolysates of casein and soy proteins are commercially available from manufacturers for use in fermentations and diagnostic media.

Alkaline hydrolysis of proteins: To the best of our knowledge, there are no reported commercial applications of alkaline protein hydrolysates in biotechnology. However, in the food industry, alkaline protein hydrolysates are used on commercial scale. Some amino acids like serine and threonine are destroyed during alkaline hydrolysis but tryptophan is intact.

Alkaline hydrolysis is a fairly simple and straightforward process; first the protein is solubilized by heating followed by the addition of alkaline agents like calcium, sodium or potassium hydroxide and maintaining the temperature to a desired set point (typical range 80–130°F). The hydrolysis will be continued for several hours until it reaches the desired degree of hydrolysis and then the product is evaporated, pasteurized and spray dried.

Enzymatic hydrolysis of proteins: Most of the enzymes used to make protein hydrolysates for applications in biotechnology are obtained from animal, plant and microbial sources. Recent advances in techniques for the hydrolysis of proteins have come from studies mainly with proteolytic enzymes from fermentation processes. The main advantages of enzyme hydrolysis of proteins is that the hydrolysis conditions are mild and enzymes are more specific enabling the manufacturers to precisely control the degree of hydrolysis and tailor make products for the end users.

A wide variety of proteolytic enzymes are commercially available from animal, plant and microbial fermentations. The most commonly used enzymes for protein hydrolysates from animal sources are pancreatin, trypsin, pepsin; plant sources are papain and bromelain and from fermentation sources, bacterial and fungal proteases. The hydrolysis of proteins can be achieved by a single enzymatic step or a sequential enzyme hydrolysis using multiple enzymes. The choice of enzyme depends on the protein source and end user requirements. For example, if the protein has a higher content of hydrophobic amino acids then the enzyme of choice should be the one that preferentially cleaves the hydrophobic amino acids (Adler-Nissen 1986).

Besides acid, alkaline and enzymatic hydrolysis of proteins, a new trend in the manufacturing of protein hydrolysates for (di and tri peptides) is by coupling of the amino acids by enzymes or mass producing them by a fermentation process (Ota et al. 1999; Pasupuleti 2005).

Generic Flow of Manufacturing Process of Protein Hydrolysates

Depending on the manufacturer and type of product, the reactor size varies anywhere between 500 and >10,000 gal. As shown in the schematic flow chart in Figs. 1 and 2, first the proteins are generally solubilized in water to anywhere between 8–20% solids. If needed the proteins are pretreated with heat (up to 200°F), acid or alkali,

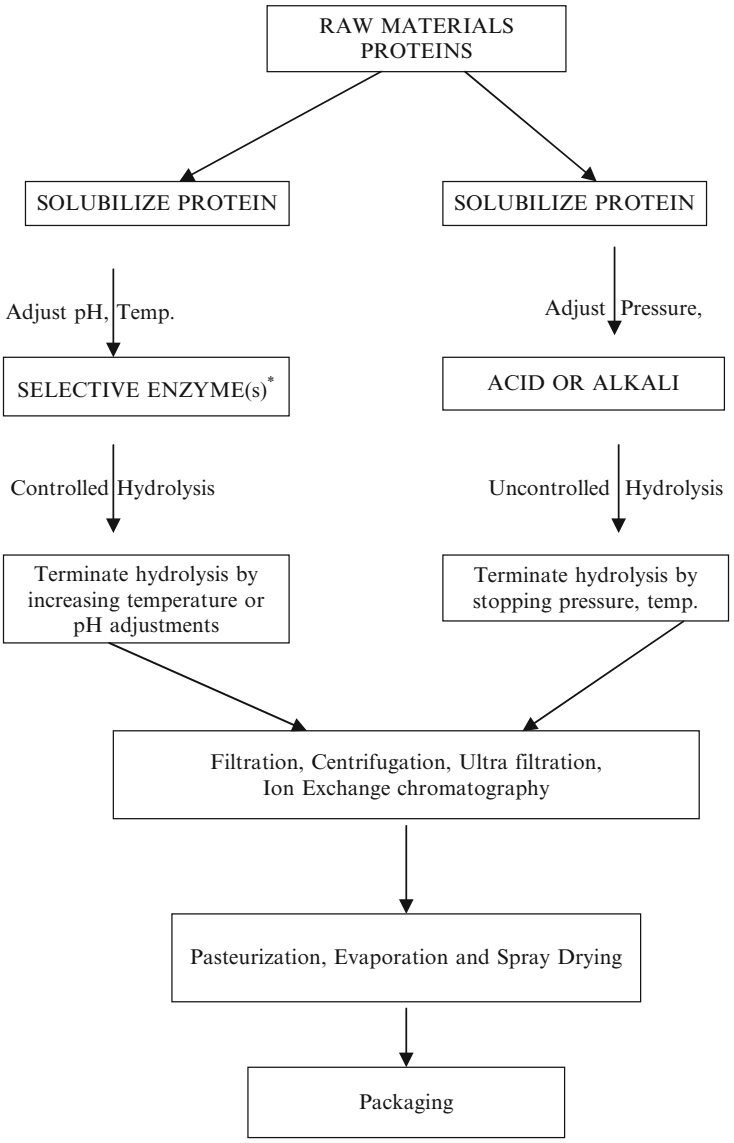

Fig. 1 Typical manufacturing overview of protein hydrolysates

Fig. 2 Bank of hydrolysis reactors (Courtesy: Kerry Biosciences)

adjusted to appropriate pH (3.5–9.0) and temperature (100–150°F) and followed by the addition of enzyme or enzyme systems. In commercial practice the enzymes used could be purified, semi-purified or crude from animal glands. The hydrolysis times vary anywhere from for 1 h to more than 100 h and some manufacturers use bacteriostatic or bactericidal preservatives especially when the hydrolysis times are longer to prevent microbial contamination. The bacteriostatic or bactericidal preservatives will be subsequently removed or evaporated during the downstream processing. Use of bacteriostatic or bactericidal preservatives is an old practice and today there are better alternatives such as UV treatment, pulse electric field applications (Uchida et al. 2008), ozone (Oliver and Duncan 2002) that need to be explored. Some protein hydrolysis manufacturers have already eliminated or are attempting to eliminate Bacteriostats in some of their processes by shortening the hydrolysis times to 4 h or using enzymes that can withstand higher temperatures or enzymes that work at acidic pH environment values. These new technologies will give better control of the process and deliver a consistent product batch after batch. The degree of hydrolysis (DH) or time in the reactor is monitored by taking in-process samples. Once the desired DH level is achieved the hydrolysis is typically terminated by heating to higher temperatures to deactivate the enzyme or enzyme systems.

Depending on end use, the protein digest is pasteurized (the term pasteurization used in this industry is different from that used in the typical dairy industry relating to time and temperature), evaporated and spray dried or more typically it goes through a series of downstream processing steps. Almost all of the products used in animal cell culture invariably go through the purification process. Typically the first step is

Fig. 3 Plate and frame filter press (Courtesy: Kerry Biosciences)

Fig. 4 Centrifuge (Courtesy: Kerry Biosciences)

to separate the insolubles from the protein digest by using a centrifuge or a plate and frame filter press or a micro filtration system (Figs. 3 and 4). Sometimes, the filtration process is repeated several times until a desirable color and clarity of

the solution is obtained. Especially when plate and frame filter press is used, an inert filter aid (usually from volcanic rocks or diatomaceous earth) is used to form a thin coating on the plates often referred to as "precoating" for better filtration. Sometimes the filter aid is mixed with the protein digest and recycles through the filter press until a nice coat is formed before the clear filtrate is collected and this process is often referred to as "body-feed coating". Charcoal powder is commonly used to decolorize and to remove haze-forming components (Chae et al. 1998; John 1993). This unit operation is very critical to obtain desirable color and clarity. The manufacturers have an arsenal of different operating conditions such as temperature (hot or cold), pH and flow rates to achieve the desired product specifications.

The latest addition to the manufacturing of protein hydrolysates is to replace the plate and frame filter press with that of the ultra filtration systems (Fig. 5). The protein hydrolysate industry typically uses spiral wound ceramic or hollow fiber membranes with a 10,000 Da molecular weight cut off. This is to remove endotoxins as a lower amount of endotoxins is preferred for cell culture applications. However, this could also potentially remove the peptides greater than 10,000 Da. For this reason some manufacturers use 50,000 or greater molecular weight cut off membranes but this may not guarantee the removal of endotoxins. Therefore, depending on the end use, the manufacturer uses either 10,000 or higher molecular weight cut off membranes. However, in a recent publication, the authors demonstrated that endotoxin specification for cell culture may not be critical (Limke 2009).

After filtration the product is typically pasteurized or heat treated to kill/reduce the microorganisms. The term pasteurization is misleading because in most cases the temperatures used are way beyond standard legal pasteurization temperatures. Some manufacturers tend to pasteurize the product multiple times. Some manufacturers tend to pasteurize the product multiple times.

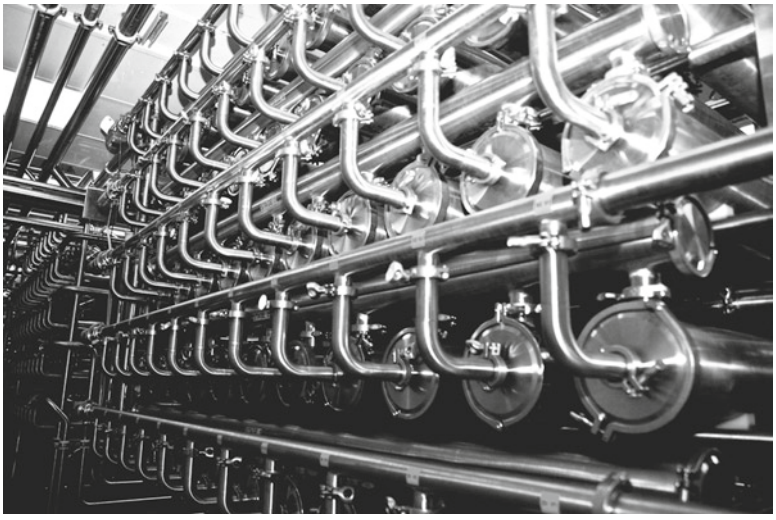

Fig. 5 Ultra filtration system (Courtesy: Milk Specialties Global)

After pasteurization the product is evaporated to remove thousands of gallons of water to bring the solids to 30–50%. Interestingly not all protein hydrolysates evaporate at the same rate and to the same level of solids. It varies from protein source, choice of enzyme and degree of hydrolysis. For this reason the bulk densities of protein hydrolysates differ.

Some manufacturers offer a concentrated protein hydrolysate, typically > 60% solids with very low water activity that is microbially stable yet dispensable through pumps. This is advantageous in terms of handling, safety and prevents the loss of protein hydrolysate as dust in batching to make the media. The other advantage is that the end user could potentially work with the manufacturers to customize the package of protein hydrolysate concentrate to their use in the fermentors. However, the end users should have the capabilities to use concentrates.

After evaporation some manufacturers go through final Pasteurization and then the product typically goes through a feed tank that feeds to the spray drier and eventually into the boxes, bags or drums for packaging.

Controlling Inconsistencies of Protein Hydrolysates During Manufacturing

The following are very important to manufacture high quality consistent protein hydrolysates:

- Strictly adhering to GMP procedures.
- Maintaining hygiene of the plant at all times.
- Screening of raw materials and qualifying vendors to obtain consistent and high quality raw materials.
- Monitoring in-process samples to maintain consistency of the batches.
- Testing finished product samples from beginning of first drum to the last drum of each batch.
- Robustness of the process dictates the consistency of protein hydrolysates.
- Constantly monitoring protein, enzyme, water sources and the downstream processing techniques as they create differences in the quality of protein hydrolysates.

As mentioned above depending on the end use, protein hydrolysates go through a variety of downstream processing steps. For example if the protein hydrolysate is specifically made for cell culture use, then typically after the hydrolysis it will go through a heating step to deactivate the enzyme followed by separation either by centrifugation or plate and frame filtration or ultra filtration, evaporation, pasteurization and spray drying. Sometimes the product fails to meet specifications especially due to color and clarity at any of these steps. When this happens the manufacturers will perform additional filtrations, adjust pH and do what is required to meet the end product specifications that are described in their standard manufacturing procedures. The goal for the manufacturer is to meet the specifications; however, by performing additional steps the quality of product may change and that

may impact the performance in end users applications. Therefore, it is important to develop a well defined and robust process that will alleviate these types of problems. This can only be achieved by partnering with the end users to understand their requirements and reviewing manufacturer's capabilities and by jointly developing physical, chemical and functional specifications.

It is also important to share the process details especially if there are any variations with the end users and they in return should share the results obtained and this will help to better define protein hydrolysates.

For example a number of practises noted below are used in protein hydrolysate manufacture but these may not be acceptable for some end uses for the reasons noted.

- When the desired degree of hydrolysis is not achieved the common practice is to add more enzymes than the standard. This could potentially change the enzyme/substrate ratio and subsequently the quality of the product.

  Sometimes this happens and the reason could be that the enzyme used from animal glands is not active. If this is the case move towards purified enzymes that have consistent activity.

- The hydrolysis times are not consistent suggesting inconsistencies and therefore it is not a robust process.

  If the enzyme activity is not consistent, add enzymes based on their activity and not by quantity.

- Goes through pH swings because of operator/machine errors.

  Validate the machines periodically and train operators.

- Performs multiple filtrations using inert filter aid and/or charcoal powder.

  Avoid this as it may potentially remove some of the amino acids and peptides. If this has to be performed make sure to check the functionality of end users' applications.

- To obtain the desired color and clarity sometimes more charcoal is added.

  Avoid this as it may potentially remove some of the amino acids and peptides. If this has to be performed make sure to check the functionality of end users' applications.

- Mixing remains of old batches of the product into the new batch during processing.

  Avoid this as it may potentially remove some of the amino acids and peptides. If this has to be performed make sure to check the functionality of end users' applications.

- Mix rejected product into the new batch in hopes to meet the desired specifications.

  Avoid this as it may potentially remove some of the amino acids and peptides. If this has to be performed make sure to check the functionality of end users' applications.

- Dry powder blend with other batches. For example, if one batch fails to meet the desired AN/TN specification of 45 and reaches only to 40. This will be potentially mixed with the next batch that is tailor made to be blended with high AN/TN 50 or greater to get to the specification of 45.

  Avoid this as it may potentially remove some of the amino acids and peptides. If this has to be performed make sure to check the functionality of end users' applications.

- The entire batch that was previously rejected for not meeting the specifications will be reworked into a new batch and sold as a new batch.

  Avoid this as it may potentially remove some of the amino acids and peptides. If this has to be performed make sure to check the functionality of end users' applications.

- Because of unanticipated problems in the processing and/or scheduling conflicts sometimes the product is held for several hours. If the hold times are unusually longer then preservatives are added which could potentially change the quality of the product.

  Avoid this as it may potentially remove some of the amino acids and peptides. If this has to be performed make sure to check the functionality of end users' applications.

All or some of the above practices may be acceptable in certain applications where the product functionality may not be very specific to protein hydrolysates. Indeed if any or all of the above is used for cell culture applications it is important that the end user understand the process used and understand the impact on the cell culture performance. Sharing this information with end-users may lead to a better understanding of protein hydrolysates and the cell culture performance. This will ultimately benefit the end user as well as the manufacturer.

Selection of Raw Materials

The most commonly used animal protein hydrolysates in biotechnology applications are casein, whey and meat obtained from different organs. Widely used plant derived proteins are from soy and wheat however; recently rice, pea and cottonseed proteins have been introduced commercially (Sheffield Product Manual 2009). It is important to note that the amino acid composition, structure of the protein, its solubility and denaturation will affect the choice of enzyme and the subsequent product.

High quality and consistent protein hydrolysates start with the selection of right raw materials. It is for this reason the manufacturers should spend a great deal of time and resources to ensure their availability, note seasonal fluctuations if any, meet regulatory requirements (BSE/TSE free, Kosher, Halal, etc.) and qualify multiple vendors.

Besides protein source, the most important raw materials are enzymes, water source and process aids as they have biggest impact on the end product. Therefore,

it is essential to thoroughly study these sources, establish rigorous quality control specifications, audit and qualify a minimum of two vendors.

Enzymes: The degree of hydrolysis (AN/TN ratio), peptide chain length, molecular weight distribution profiles, free and total amino acids are dependent on the choice of enzyme or enzyme systems.

Compared to acid and alkaline hydrolysis of proteins, proteolytic enzymes offer significant advantages such as:

- Requirement of small amounts of enzymes that can be easily deactivated after the hydrolysis.
- Mild operating conditions like temperature and pH.
- No destruction of amino acids.
- No production of chloropropanols.
- Hydrolysis of certain amino acids resulting in less complex mixtures of peptides that can be relatively easily purified.
- Availability of several choices of enzymes. This enables the manufacturer to pick the best one to specifically modify the proteins that best suits end-user applications.

The use of proteolytic enzymes or proteases that specifically break down the proteins into peptides with different peptide chain lengths and free amino acids has been in practice for more than several decades. In the beginning, most of the proteases used to hydrolyze proteins for use in the growth of microorganisms were from animal sources especially porcine. Interestingly enough, even today these enzymes are widely used individually or in mixtures. Some of the common examples are pepsin, pancreatin, carboxy peptidases and amino peptidases. At the same time, proteases from plant sources are also widely used, for example, papain and bromelain. Fermentation technology and genetic engineering disciplines have advanced over the years and yielded novel enzymes. Few examples, serine proteases, fungal proteases, endo and exo peptidases.

The industrial practice of defining protein hydrolysates is by determining the degree of hydrolysis of proteins which is the percentage of peptide bonds cleaved form a given protein; refer to Chapter 1 for the definition of degree of hydrolysis. The protein hydrolysates used in biotechnology are often described by the ratio of Amino Nitrogen to Total Nitrogen (AN/TN). The most commonly used methods to determine AN are by:

1. Formal Titration (Sorensen 1908) Opa (H. Frister, H. Meisel, E. Schlimme)
2. OPA method modified by use of N,N-dimethyl2-mercaptoehtyl ammonium chloride as thiol component (Fres 1988)
3. TNBS (Adler-Nissen 1979)

The total nitrogen is determined by traditional Kjeldahl (Bradstreet 1954) or modified Kjeldahl (Fearon 1920) or combustion methods (Brink and Sebranek 1993).

Further, protein hydrolysates defined by determining the amount of free and total amino acids; molecular weight distribution of peptides (Jandik et al. 2003; Jun et al. 2008).

End Use of Protein Hydrolysates Dictates the Downstream Processing

Choosing the right protein, enzyme(s) and obtaining the desired degree of hydrolysis is only half the battle. Equally important is the downstream processing as it dictates the end use of protein hydrolysates. For example, after protein hydrolysis, it goes through one simple separation step and the resulting product may not be clear and soluble. This product will not meet the demands of dehydrated culture media or cell culture applications but may be good enough as a nitrogen source for some industrial fermentations. Similarly a product that did not go through ultra filtration with the 10,000 molecular weight cut off and was neither monitored nor controlled for endotoxins may not be good for certain cell culture applications.

Some of the commonly used equipment for purification are:

- Centrifugation
- Plate and Frame Filtration
- Micro Filtration
- Ultra Filtration
- Nano Filtration
- Ion Exchange Chromatography

The detailed description of most of the above equipment is beyond the scope of this chapter and can be found in the literature as they are commonly used in biotechnology. However, a brief description of plate and frame filtration will be given to make the reader familiar with it, how and why it is still commonly employed by some of the protein hydrolysate manufacturers.

Plate and Frame Filtration

Essentially plate and frame filter presses are dewatering machines that work under pressure typically at 60–80 psi to remove the insolubles from protein hydrolysate digests. They are particularly suited in cases where insoluble solids are higher that may potentially plug the micro and ultra filtration membrane systems.

Typically diatomaceous earth is used with plate and frame filters to aid in the filtration of very fine solids. This coating is first applied to the filter, allowing an even coating of the filter cloth; then the feed is introduced into the press or sometimes the diatomaceous earth is mixed with the protein hydrolysates slurry and run through the filter until a fine coating is formed. During this time the filtrate is recycled back to the protein hydrolysate digest. This will typically take about 15–30 min and once the coating is formed, the filtrate will be collected. The biggest advantage of using the plate and frame filter is that it allows removal of color during filtration by treating with various grades of carbon. However, it is important to pay attention to the type and quantity of carbon applied as it may potentially remove some of the peptides and amino acids (Silvestre et al. 2009).

Figure 3 shows the basic operation of a plate and frame filter press. The feed enters the filter press at the bottom of the plate, using a pump that is capable of creating 80–90 psi. Then, the feed travels the path of least resistance (up between the filter plates, which has filter media inserted between the plates), and the void between the plates is filled with the slurry, as the liquid passes through the filter media, and travels up to the outlet port at the top of the plate. This liquid is referred to as the "filtrate", and is discharged from the filter press. The solids remain in the void between the plates, until the plates discharge the filtered solids (Mine Engineer Product Manual 2009).

Large plate and frame filter presses have mechanical "plate shifters", to move the plates, allowing the rapid discharge of the solids stuck in between them. Also, they have the capability of blowing compressed air into the plate, to dry the cake, and collect the filtrate as much as possible. Typical capacities for a plate and frame filter will depend upon the solids being dewatered. However, they will range around 1/2 gal per min of feed for low solids content slurries (<1% solids) to over 1 gal per min of slurry per square foot of surface area on the plates. For example, a 50 ft^2 filter press would dewater 25 GPM of 1/2% solids feed, or about 50 gpm of 10% solids feed (Mine Engineer Product Manual 2009). The volume between the plates will dictate how often the filter press should be stopped in order remove solids from the plates. Solids removed from the filter press typically fall into a hopper or directly onto a conveyor belt for further transport to the next stage of the operation. The filtrate goes through a series of downstream processing steps depending on the end use application. A 10 ft^3 volume press would hold 10 ft^3 of dewatered solids.

Many protein hydrolysate manufacturers first use a laboratory or small scale pilot plant plate and frame filter press to determine and a optimize the filtration in terms of color, clarity, throughput and yields. The smaller versions are exactly like a full scale press, and results obtained can be scaled up for plant operations, accurately.

Depending on the end use and manufacturers capabilities, sometimes after the first plate and frame filtration, the filtrate is passed through micro and ultra filtration systems. It is also a common practice of some manufacturers to use plate and frame as the last filtration step to obtain the desired color and clarity. In cases where very low salts are desired, the filtrate may go through ion exchange chromatography to remove the salts.

Different Methods of Concentration and Drying

Several methods are employed to concentrate the protein hydrolysates such as falling film, rising film evaporators. In some instances nano filtration is also used to concentrate (Fig. 6).

Depending on the nature of protein hydrolysates, they are concentrated from 25–>50% solids before drying. In some instances, the manufacturers may not dry

Fig. 6 Falling film evaporator (Courtesy: Niro Inc.)

and offer protein hydrolysates in a concentrate form that is pumpable at > 60% solids. The water activities of these concentrates are kept low so that they are microbially stable.

Once the product is concentrated, it is pumped to a feed tank that goes through a cartridge filter to remove any larger particles, then to a high pressure pump to feed the dryer. Spray driers are widely used in the industry and some manufacturers use roller drum driers (Figs. 7 and 8).

Considerations for Plant Design and Layout

Most of the protein hydrolysates manufacturers have been in the business for several decades and so are their plants. They are very good in adding new equipment to stay up with the latest technology and to increase productivities.

Whether it is an existing or a new plant, the most important things to consider are safety, hygiene, water source. If needed treat the water with appropriate technologies such as reverse osmosis or water softeners. Use proper drainage systems, handling of solid and liquid effluents and right size tanks with appropriate design for mixing. If direct steam is used to heat the tanks, they should be able to withstand steam injection. It is important to use downstream processing equipment to match the size of the tanks, strictly complying with GMP procedures.

Since protein hydrolysates are made from a wide variety of proteins including meat and animal tissues, it is important to determine if the vendors can provide documentation and answer customers' questions on regulatory issues. If yes, to what extent is the support available; and the certificate of suitability from the European Commission.

Fig. 7 Spray dryer (Courtesy: Niro Inc.)

Concerns of the Biotechnology Industry for Regulatory Support from Protein Hydrolysate Manufacturers

- Is there adequate separation of animal origin products from that of the vegetable proteins?
- Are the raw materials and finished products appropriately labeled and stored to prevent mix-ups?
- How are the raw materials and finished products that did not pass the QC tests segregated?
- Are discrepancy reports issued for the products that did not meet the required specifications and written procedures describing how this product is disposed of?
- Is there a quarantine location to hold the products before QA/QC releases the raw materials and finished product?
- Are there written acceptance specifications for raw materials and finished products?

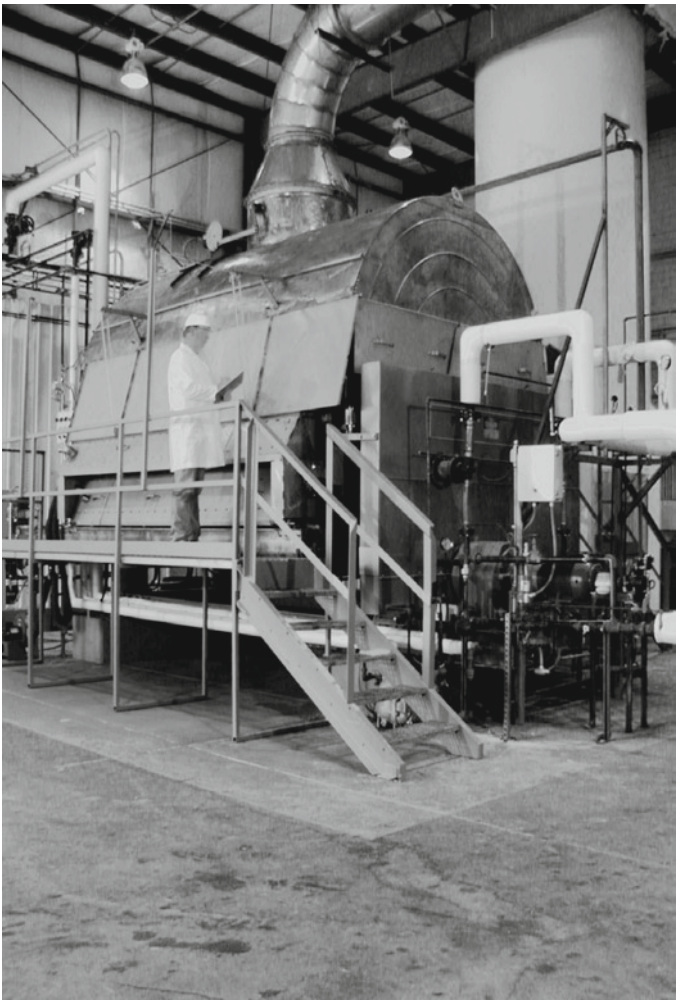

Fig. 8 Drum dryer (Courtesy: Nutraflo Protein Products)

- How the cross contamination between animal origin and vegetable proteins is prevented?
- Are there good written cleaning procedures in place?
- Are the cleaning procedures validated and documented?
- What measures are taken to ensure that the cleaning agent is completely removed from the process tanks, lines and equipment?
- If preservatives are used in the manufacturing, what procedures are followed to ensure that they are totally removed from the product?
- What tests are performed to ensure that the enzyme is totally deactivated?
- Does QA/QC report to somebody other than manufacturing?
- Is the process reproducible and validated?
- Is the process equipment calibrated and validated?

- Are there written Manufacturing Standard Operating Procedures?
- Is the water periodically tested and are there written specifications?
- Is the manufacturing plant designed to control bioburdens, endotoxins and mix-ups with other products?
- What is the lot size and is it produced by following the approved written production process and formulations?
- Are the test procedures validated and documented?
- Are instruments calibrated by following a written schedule and against standards traceable to the NIST?
- Is the process equipment sufficiently cleaned, tested and documented so that the product from previous runs is not getting mixed up?
- Is the temperature and humidity of the warehouse monitored constantly and documented?
- Change control procedures – who is responsible?

Specifications and Sampling

It is very important that the specifications and sampling should be relevant to the end users and should match the capabilities of the manufacturers.

Typically, protein hydrolysate manufacturers will take in-process and finished product samples to ensure that the product is in compliance with the established specifications for that particular product.

It is important that these specifications are relevant to your applications. The most important aspect of sampling is how representative the sample is of the entire lot. The lot varies anywhere from 1,000 to >10,000 pounds. There is a possibility that the product from container #1 could be different from the last container because of the inherent long processing times. Therefore, it is reasonable to ask the manufacturer about sampling plans and ensure that a representative sample of the entire lot is taken. At a minimum, ask manufacturers to take samples from the first, middle and the last drum and blend them together before use.

If the sample is not uniform with the whole lot, then the chances are that the customer may get poor results with their trials and may not trust the data.

What specifications and test methods should be established for cell culture?

It is critical to minimize the variations and deliver consistent protein hydrolysate batch after batch.

For recommended tests, it is important that both the laboratories should follow the same test method to avoid confusion. Important test methods are:

- Solubility; define appropriately and perform the test at 50°C.
- Clarity with 5% solids using Hach turbidometer.
- Color with 2% solids at 420 nm.
- Filterability with Vmax filterability test method.
- Endotoxins with Gel clot method and reported as EU/g (Tsuji et al 1980).

- AN with Sorensen or Formaldehyde titration method or OPA or TNBS method.
- TN with Kjeldahl or direct combustion method.
- Minerals with atomic absorptiometer.

These are only a few guidelines and the end users have to customize the specifications and test methods to suit their needs. Clearly communicate and get agreement with the protein hydrolysate manufacturers.

Manufacturing capabilities should go beyond meeting the product specifications. As in some applications, for example in cell culture, protein hydrolysates significantly enhance the yields but its mechanism is not clearly established. In such cases, it is essential that the manufacturing of protein hydrolysates be highly consistent with respect to the process starting from raw materials, pretreatment, hydrolysis times, filtration, pasteurization, evaporation, spray drying times to the packaging. Clear understanding of manufacturing capabilities in meeting the product specifications and understanding end users' applications is important. Sharing knowledge and collaborating with each other will lead to better products and increased productivities.

Future Trends

Continuous Process

To the best of our knowledge, all the protein hydrolysate manufacturers employ traditional batch systems. However, there is a potential to use immobilized enzyme or membrane bioreactor systems to save the enzyme cost and increase the productivities (Holownia 2008).

Innovative Technologies

Manufacturing of protein hydrolysates, peptides by fermentation process. An example is glutamine dipeptide. Potentially this technology could be used to make a variety of dipeptides and oligopeptides (Yagasaki 2009). The other example is manufacturing of gelatin hydrolysates by a fermentation process in response to the BSE problem (see Chapter 12 by Olsen et al. 2010).

Another revolutionary technology is the enzyme coupling method to manufacture peptides. The amino acid is esterified, and the ester is enzymatically coupled with another amino acid to make a dipeptide and so on. Conventional production methods require complicated production processes that often produce a high level of impurities (Ajinomoto Product 2009).

We have already seen a shift in using more and more enzymes obtained from fermentation process. This ensures that the enzyme activity is well defined. Enzyme

manufacturers can tailor make enzymes to deliver specific protein hydrolysates for a specific function.

New equipment such as electro dialysis, nano filtration may have potential use for manufacturing protein hydrolysates (Orue 1998; Laurant and Loubna 2009).

Non-dusting protein hydrolysates may play a key role in the future as it eliminates or minimizes dust.

Collaboration and Partnerships

One of the essential elements in successful manufacturing of protein hydrolysates for biotechnological applications is working as a team in partnership with the manufacturers as well as the end users.

This type of partnership may result in new and innovative products. For example, there is a distinct possibility that protein hydrolysate manufacturers may develop one single product that may help the end users eliminate maintaining a "laundry list" of several media ingredients. They may even customize the protein hydrolysates along with other media ingredients to fit the end user fermentor size.

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http://www.springer.com/978-1-4020-6673-3

Protein Hydrolysates in Biotechnology
Pasupuleti, V.K.; Demain, A.L. (Eds.)
2010, XIII, 229 p., Hardcover
ISBN: 978-1-4020-6673-3