Review Article

Recent developments with debittering of protein hydrolysates

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Abstract

The products of protein hydrolysis, which are normally mixtures of peptides and amino acids, are called protein hydrolysates. Food protein hydrolysates have a wide range of nutritional applications. The production and use of protein hydrolysates differ in the type, level of hydrolysis and the peptide profile. Bitterness in protein hydrolysates is associated with the release of peptides containing hydrophobic amino acid residues which is one of the major undesirable aspects of protein hydrolysates for various applications. Numerous options have been investigated in the debittering of food protein hydrolysates. These include solvent extraction, adsorption of bitter peptides on activated carbon, adsorption onto matrices, chromatographic removal using different matrices and selective extraction with alcohols and treatment with exopeptidases, addition of polyphosphates, specific amino acids,T- plastein. Thus the various methods for the debittering of protein hydrolysates and their pros and cons are expounded here.

Keywords: peptides, amino acids, food, nutrition, India.

Introduction

As well as its primary function of supplying nutrients and energy, protein also has numerous tertiary functions relating to physiological regulation. There are four ways to get amino acids into the bloodstream: 1) whole food proteins: 2) intact protein supplements, 3) free form amino acids, and 4) protein hydrolysates [1]. Hydrolysis of proteins involves the cleavage of these peptide bonds, resulting in breakdown of proteins to peptides and amino acids. These products of protein hydrolysis, which are normally mixtures of peptides and amino acids, are called protein hydrolysates [2].

Why Hydrolyze Proteins? The Importance of Protein Hydrolysates

Various physiological activities have been found in the hydrolysates derived from the proteolytic hydrolysis of various food proteins [3]. Protein hydrolysates have been used since the 1940's for the nutritional management of individuals who cannot digest intact protein [4, 5]. Protein hydrolysates form an important ingredient of medical diets for various ailments and are gaining acceptance as components of sports as well as weight control diets. Hydrolysis also results in a reduction in allergenic potential by the destruction of allergenic epitopes. Significant current interest exists in the hydrolytic release of bioactive peptide sequences encrypted within the primary structure of food proteins [6]. Protein hydrolysates are administered normally in clinical nutrition for Phenylketonuria (PKU). Moreover, many of the protein hydrolysates were found to possess antioxidant activity and Angiotensin I-Converting Enzyme Inhibitory activity [7, 8]. Protein hydrolysates are also useful as a nitrogen source in the growth media for microorganisms [9, 10].

Dairy proteins can be hydrolyzed to modify their functional properties [11]. For example, hydrolysis of casein can improve its solubility at pH range close to its isoelectric point so that it can be used in applications requiring high solubility in low pH range. Whey proteins, which are normally not very stable to heat treatment in many food processes, can improve their heat stability through enzyme hydrolysis [12, 13]. Several studies have shown that protein hydrolysates containing mostly di- and tripeptides are absorbed more rapidly than free form amino acids and much more rapidly than intact proteins [14].

Production of Protein Hydrolysates

Protein hydrolysates are produced from purified protein sources by heating with acid or preferably, addition of proteolytic enzymes, followed by purification procedures [15]. Hydrolysis decreases the peptide size, making hydrolysates the most available amino acid source for protein biosynthesis [16]. The most commonly used proteins for producing food grade hydrolysates include casein, whey and soy proteins. Enzyme hydrolysis is strongly preferred over strictly chemical methods for producing hydrolysates in nutritional applications [17]. Enzyme-catalyzed processing is very specific and can be controlled to produce protein hydrolysates of defined molecular weight distribution, peptide composition, and degree of hydrolysis (DH) and carried out under mild conditions [18]. Acid and alkali hydrolysis, on the other hand, can destroy L-form amino acids, produce D-form amino acids and can form toxic substances like lysino-alanine [17]. Especially, acid hydrolysis oxidizes cysteine and methionine, destroys some serine and threonine and converts glutamine and asparagine to glutamate and aspartate, respectively, lowering protein quality and biological value [15]. Hydrolysis of proteins by proteolytic enzymes (often referred to as proteases) is a widely used technique to modify the physicochemical properties (e.g., molecular size, surface activity/hydrophobicity, etc.), functional properties (e.g., gelation, emulsification, solubility, etc.) and sensory properties of food proteins [11]. Enzymatic hydrolysis of proteins produces a decrease in peptide size, which can modify functional characteristics of the proteins and improve their quality [19]. After the substrate (i.e., protein) and enzyme(s) selection for enzyme-catalyzed processes, the DH is the principal variable to be used in optimization [20], to achieve desired protein hydrolysates. One of the major elements in commercial production of hydrolysates is developing the optimal enzyme mixture for producing the desired ratio of amino acids, dipeptides, tripeptides and oligopeptides [17].

Several proteases can be used potentially for making hydrolysates. Some of these proteases are site specific enzymes. Because of their specificity, the types of polypeptide fragments released in the hydrolysate differs between proteases. Alcalase from *Bacillus licheniformis* is a major commercial

enzyme used in the manufacture of protein hydrolysate. This enzyme belongs to the family of subtilisins, which are serine proteases.

Fundamentals of Protein Hydrolysates

The proteases which hydrolyze peptide bonds from the N-terminus or C-terminus, one amino acid residual (sometimes two residuals) at a time, are called peptidases or exopeptidases. The proteases which hydrolyze peptide bonds in the interior of the polypeptide chain are called proteinases or endopeptidases. Based on the mechanism of catalysis, proteinases can be divided into four subgroups: serine proteinases, cysteine proteinases, aspartic proteinases and metallo-proteinases. Some proteases have a very specific requirement for R1 or R2 to hydrolyze the peptide bond. Such requirements are referred to as the substrate specificity of the protease. For example, trypsin only hydrolyzes the peptide bond when R1 is the side chain of an arginyl or lysyl residue. Therefore, trypsin has a narrow specificity. Some other proteases have broader specificities. An example is Subtilisin A, which hydrolyzes peptide bonds when R1 is the side chain of a hydrophobic amino acid residue [2]. The rate of protease catalyzed hydrolysis of proteins generally depends on the types of substrates, enzyme and substrate concentrations, pH, and temperature [2].

Equation (1) illustrates an enzyme-catalyzed hydrolysis of a peptide bond. At high pH, hydrolysis of peptide bonds results in the release of protons; the uptake of protons results at low pH [2].
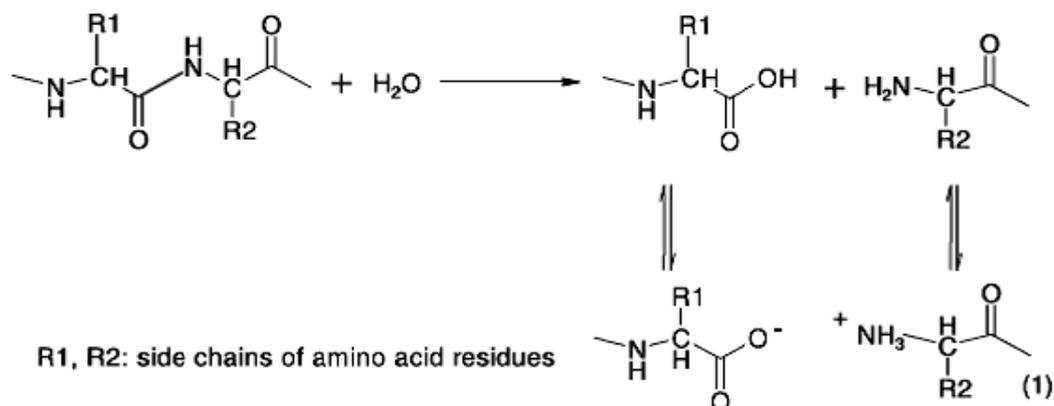
Eq(1) Enzyme-catalyzed hydrolysis of a peptide bond [2].

Analysis of Protein Hydrolysates

Depending on the uses of protein hydrolysates, they can be characterized by their level of hydrolysis, peptide profile, functional attributes, and biological activities. Trichloroacetic acid (TCA) solubility has been used as a measure of hydrolysis level since as a protein is further hydrolyzed, smaller peptides and amino acids, which are more soluble in TCA solution, are formed [21]. The functional properties of the protein hydrolysates is dependent upon the degree of hydrolysis (DH) and the physiochemical properties that is, size, solubility, etc., of the polypeptides in the hydrolysate.

The **DH** is defined as the fraction of the peptide bonds cleaved and is often expressed as a percentage.

$$\% \ DH = (n/n_T) \times 100$$

Where n_T is the total number of moles of peptide bonds in one mole of protein and n is the number of moles of peptide bonds cleaved per mole of protein.

The DH is generally monitored by the pH –Stat method. Several methods exist in determining the amino groups in a hydrolysate. Formol titration of amino groups is one of the well-known methods. It is based on the fact that in neutral or alkaline solutions, the amino groups can react with 2 moles of formaldehyde and form a product with much lowered pKa (Eq. 2) [22, 24].
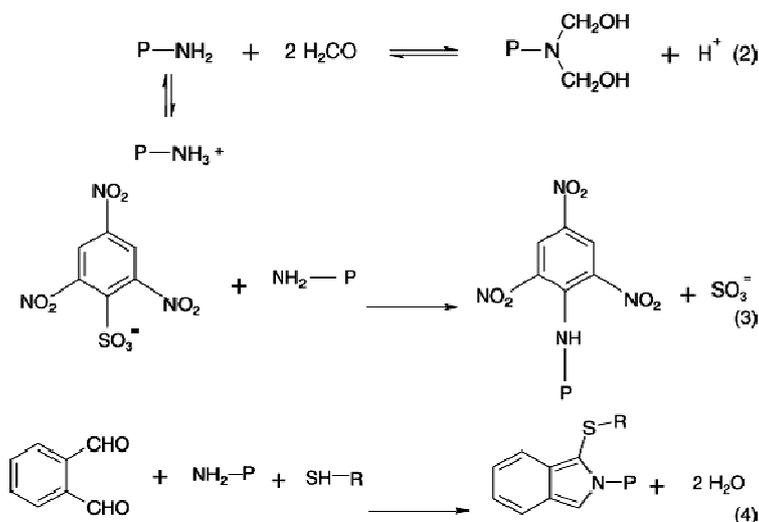
Eq (2, 3 & 4) Formol titration of amino groups, estimation of amino groups [23, 24].

The ratio of amino nitrogen to total nitrogen (AN/TN) also reflects the hydrolysis level. For any protein, there is a linear relationship between AN/TN and DH. Using size-exclusion chromatography (SEC) on HPLC, the peptide profile (molecular weight distribution) of protein hydrolysates can be determined [25, 26, 27]. For some medical and nutritional applications of protein hydrolysates, the presence of immunoreactive peptides can be detected Enzyme-Linked Immunosorbent Assay (ELISA) and Radio Allegro Sorbent Test (RAST) [28, 29].

Bitterness in Protein Hydrolysates

One of the attributes that reduces the consumer acceptance of protein hydrolysates is the bitterness caused by the presence of low molecular weight (>10 kDa) peptides containing pro, leu, tyr, phe, ala, etc., in specific combinations [30]. Intact food proteins do not display bitterness as their molecular size militates against their ability to interact extensively with bitterness receptors in the oral cavity. Bitterness in protein hydrolysates has been classically associated with the release of peptides containing hydrophobic amino acid residues. The bitterness is one of the major undesirable aspects of protein hydrolysates for various applications, particularly for beverages [31]. The bitterness of peptides increases with the increasing hydrophobicity of the C-terminal residue, where a basic amino acid, or a hydrophobic amino acid was located at the N-terminal position [34]. Intense bitterness is also associated with peptides having at least two hydrophobic amino acids at the C-terminal. In addition, peptide bitterness may increase with the number of Leu, Phe and Tyr residues [35].

It has been hypothesized that the degree of hydrophobicity was the most important predicator of the peptide bitterness and thus proposed the **Q rule** [30, 35], that was defined as the sum of the free energies of transfer of the amino acid side chains from ethanol to water, divided by the number of amino acid residues in the peptide [36]. It was reported that peptides with hydrophobicity (Q) values >1400 cal per mole and molecular masses < 6 kDa display bitterness. However, peptides with Q values <1300 cal per mole along with peptides with Q values > 1400 cal per mole and molecular masses > 6 kDa would not be bitter. Therefore, peptides < 6 kDa having a high content of Leu, Pro, Phe, Tyr, Ile and Trp residues are likely to be bitter [36]. Thus the presence of internally sited hydrophobic amino acid residues leads to greater bitterness than when the hydrophobic residues are located at either the N- or C-terminus in peptides [37]. The presence of internally sited Pro residues was shown to be a major and distinct contributor to peptide bitterness due to the unique conformation associated with this amino acid [34].

Quantification of Hydrolysate/Peptide Bitterness

The ideal route for quantification of hydrolysate bitterness is to use sensory evaluation panels [38]. This is a very time consuming activity requiring an appropriate number of panelists and training to detect bitterness in order to obtain statistically relevant data. However, instrumental methods which distinguish hydrophobic peptides within hydrolysates such as reversed-phase chromatography and Fourier transform infrared (FTIR) spectroscopy in combination with multivariate data analysis may find application in fingerprinting bitterness in hydrolysates.

Debittering of Protein Hydrolysates

Numerous options have been investigated in the debittering of food protein hydrolysates. These include:

    1) Solvent extraction [39],
    2) Adsorption of bitter peptides on activated carbon, adsorption onto matrices [40],
    3) Chromatographic removal using different matrices and
    4) Selective extraction with alcohols [41] and
    4) Treatment with exopeptidases [35].

These procedures, however, lead to the loss of some amino acid residues from hydrolysates.

Bitterness can also be masked in hydrolysates via
1) The addition of polyphosphates, specific amino acids such as Asp and Glu, α-cyclodextrins and by the admixture of hydrolysates with intact protein samples [42].
2) T- plastein reaction in addition to cross-linking using transglutaminase [43].
However, extensive hydrolysis of the protein substrate destroys the functional and biological activity of the peptide of interest. In addition soapy and brothy off-flavours often develop. A final disadvantage is that for some hydrolysates the bitterness is only partially removed..

Debittering by Solvent Extraction

Extraction of enzymatic protein hydrolysates with azeotropic secondary butyl alcohol (SBA) or aqueous ethanol (AE) or aqueous isopropranol (AI), seems to be an efficient and generally applicable method for removal of bitter compounds. The bitter peptides are concentrated in the alcohol-phase

which has an extremely bitter taste. It has a concentration of 40-70% essential amino-acids. In the alcohol-phase leucine, isoleucine, phenylalamine and tryptophan were particularly increased [44].

Debittering by Lysine-Acetylation of Soy Protein Hydrolysate

In an attempt to reduce the bitterness of soy protein hydrolyates, chemical modification of lysine was carried out by treating isolated soy protein (ISP) with N-acetylimidazole for acetylation of lysine and tyrosine. The lysine-acetylated soy protein and control protein were hydrolyzed by bromelain to the same degree of hydrolysis (10%), and then bitterness of each protein hydrolysate when compared indicated that bitterness of hydrolysates of lysine-acetylated ISP decreased in comparison with hydrolysates of control ISP [45].

Debittering by Plastein Formation

Plastein is an insoluble polypeptide formed through the random condensation of amino acids or peptides under the catalytic influence of a proteinase-like chymotrypsin; molecular weights as high as 500,000 Da are reported. These physical properties could be useful in the food industry, where the reaction might find a role in the preparation of thickening or gelling agents in products such as novel desserts, for example. The plastein products form thixotropic gels or thixotropic viscous solutions, depending on concentration, which are stable over a very wide pH range and quite a broad temperature range. However, it is essential to improve the economics of plastein formation very considerably by understanding more about the reaction mechanism [43]. Experiments showed that the bitterness of the alcohol-soluble fraction could be reduced by applying the plastein reaction [44]. However, under certain conditions the formation of plastein produces certain toxic substances [46].
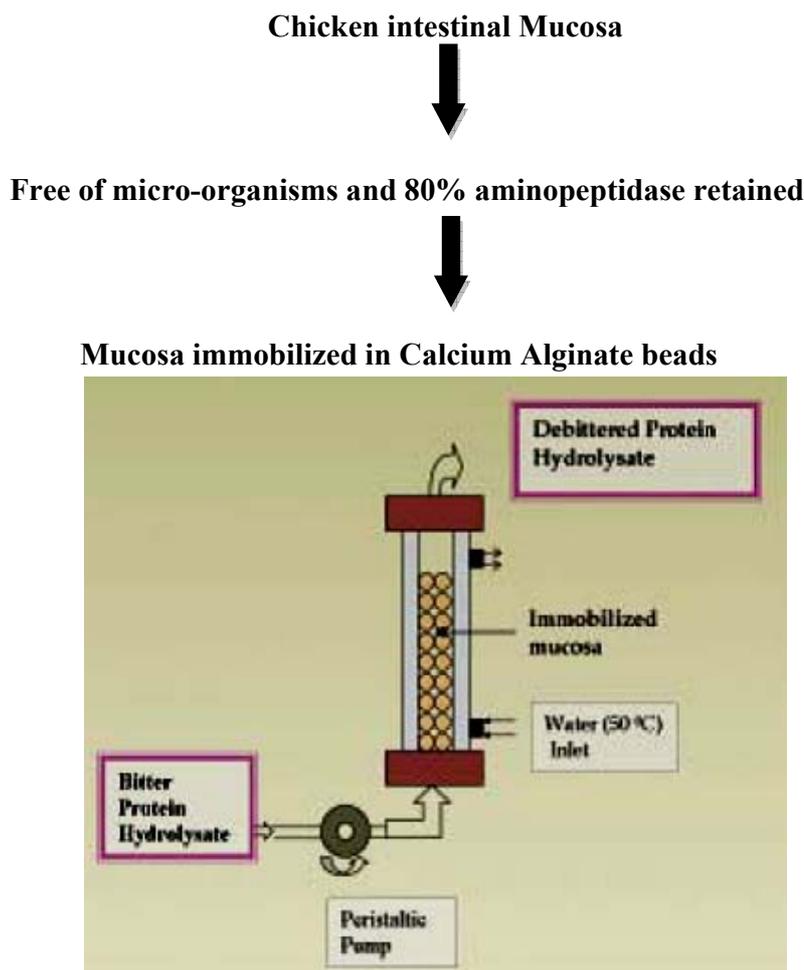
Enzymatic Debittering

Arai *et al*., [47], reported that the combination of aspergillopeptidase A and *Aspergillus* acid carboxypeptidase was capable of having both a deodorization and a debittering effect to produce a bland soybean protein hydrolyzate. It was reported that wheat carboxypeptidase was also able to eliminate the bitter taste in the peptic hydrolyzate of soybean protein [48]. Enzyme modified cheese (EMC) supplemented with Neutrase®0.5L (neutral protease derived from *Bacillus subtilis*), tends to induce accumulation of intense bitter peptides [49, 50], and this can be reduced with the crude enzyme extract from *Lactobacillus casei* ssp. *casei* LLG [51]. X-prolyl dipeptidyl peptidase activity has been detected in the crude extract of *Lactobacillus casei* ssp. *casei* LLG [52]. The bitterness of the soybean protein hydrolysates produced by the Alcalase 2.4L decreased when the hydrolysates were treated together with the *A. elegans* extract [53].

Immobilization System for Debittering Protein Hydrolysates using Chicken Intestinal Exopeptidases

Enzymatic debittering, though considered technologically and nutritionally superior, possesses limitations such as lack of economic viability and availability of broad specificity enzymes. Chicken intestine, a poultry processing waste, is a rich and viable source of potential enzymes and is considered to be a good alternative for an inexpensive immobilization system for debittering protein hydrolysates using chicken intestinal exopeptidases [54, 55]. Entrapment in Ca-alginate serves as an easy method for the immobilization of chicken intestinal aminopeptidases. There is no leakage of mucosal enzymes from the immobilized matrix. More importantly, aminopeptidase in the

immobilized system exhibits broader temperature and pH optima as compared to the free mucosal aminopeptidases. Immobilization also facilitates the stability of aminopeptidases. The debittering capability of the immobilized system is apparent from the organoleptic studies. Debittering of protein hydrolysates can be effected by a single pass of the hydrolysate through the immobilization system. Flow rate can be adjusted so as to maintain a contact time of approximately 1h between the hydrolysates and the beads. The immobilization system for the debittering of protein hydrolysates is illustrated in Figure 1.

Chicken intestinal Mucosa

Free of micro-organisms and 80% aminopeptidase retained

Mucosa immobilized in Calcium Alginate beads

Figure 1. Immobilization system for debittering of protein hydrolysates [54].

Decrease in bitterness could be ascribed to the degradation of hydrophobic bands as evidenced by the clear shift in RP HPLC profiles towards hydrophilic regions. Such a shift in the RP-HPLC profiles has been shown to be a characteristic feature of debittering. The amino acid composition of both the hydrolysates was found unchanged on passing through the immobilized column and the results are given in Table 1 [54]. Continuous operation of the process can be performed for over 3 days with a slight change in the efficiency of the system.

Some advantages of the system are:

 ➢ Presence of multiple exopeptidases capable of acting on all the amino acid residues,

> High rates of catalytic efficiency and stability over a wide range of temperatures and pHs encountered in food industry operations [56].

However the limitations of the system are:-

> Maintaining the system under aseptic conditions

> Potential of the hydrolysates to support microbial growth at enormous rates

Table 1. Amino acid profile of protein hydrolysates after treatment [54].

AMINO ACID	CASEIN HYDROLYSATE	SOYBEAN HYDROLYSATE
Asp	95.61	91.65
Glu	111.27	100.28
Ser	97.25	86.13
Gly	96.92	90.40
Thr	96.97	90.87
Arg	113.25	96.72
Tyr	97.29	92.69
Val	104.76	99.88
Met	96.10	97.42
Ile	105.05	98.35
Phe	100.24	92.19
Leu	97.07	99.19
Lys	97.74	93.99
Values are expressed as % amino acid content of untreated samples		

Debittering of Hydrolysates by Adsorption

The debittering of hydrolystaes by adsorption is complicated, time-consuming, expensive and also proves to be less efficient. This process involves passing the hydrolysates through a bed of phenolic resins [57]. It is of utmost importance to optimise the precious hydrolysate nitrogen in protein hydrolysates and reduce the cost of hydrolysates processing.

Proteins have been demineralised/desalted using either nanofiltration membranes [58], or gel permeation chromatography using the desalting SephadexTM gels (G-10, G-25 and G-50) as well as the specialist HiTrapTM desalting columns, which are not inexpensive. A process of separating bitter protein fraction separated from the feed source [59], uses Octadecyl-Siloxane $(R_2SiO)_n$ which is an analytical separation media used for chromatography separations mainly used to separate peptides from aqueous solutions. This process generates a non-bitter fraction of hydrolysate. The siloxane used can be easily regenerated. Further, this method helps to produce protein hydrolysate which has got a very low antigenicity [59].

Selective Removal of Hydrophobic Peptides from Protein Hydrolysates in a Continuous Supported Liquid Membrane Process

The removal of hydrophobic peptides from a casein hydrolysate solution was done through a supported liquid membrane (SLM) in a continuous flow system. The following conditions were found favourable for the selective removal of peptides: 1) a feed solution pH of 4.5, 2) a dilute feed concentration (<20 g/L), 3) slower feed and strip flow rates (<20 mL/h), 4) a liquid membrane prepared with 10-20% carrier, and 5) a regeneration scheme after every 24 hours [60].

Debittering and Desalting Protein Hydrolysates using Macroporous Resin

Tremendous progress has been made in ligand chemistry and various cheap resins are currently available. They are cheaper to produce, are easy to use aside from the added advantage that they can be regenerated easily and completely for re-use. Macroporous adsorption resins (MARs) are non-polar adsorbent resins that have been used for desalting biological samples, casein non-phosphorylated peptides [8, 61], and taurine from industrial manufacture [62], with good hydrolysate recoveries. The interaction with the hydrolysates are classically hydrophobic in nature [61], being favoured by high temperature and low pH. In this way desalting and separation of peptides based on their selective propensity to adsorb hydrophobically to the resins may be exploited.

The desorption of DSPH peptides from the MAR showed that the interaction between the resin and the DSPH was indeed hydrophobic in nature, because even though alcohol had both hydrophobic and hydrophilic zones, the hydrophobic zone was in greater part [41]. The desorbtion of the hydrolysates from the MAR was done with 25, 50 and 60% AC (alcohol concentration) but the 25% AC was observed to have extracted the DSPH that were not bitter while 50% AC was moderately bitter and 60% AC was significantly bitter [63]. The protein content of the DSPH obtained from AC 25% increased from 93.89 to 96.15%, AC 50% increased from the same 93.89 to 97.03%, but there was a decrease for the AC 60% extracts (93.89 to 91.17) [63]. Protein hydrolysates inhibiting the ACE *in vitro* are potentially interesting constituents of blood pressure reducing products. It was also observed that the fraction AC 60 contained the highest amounts of Methionine, Valine Alanine, Isoleucine, Leucine, proline, tyrosine and phenylalanine with the highest content of hydrophobic peptides.

Even though AC 60 was bitter, it however showed the desirable property of being a better ACE inhibitor, which could make it useful in other applications especially in the pharmaceutical industry [63].

When the products were compared with the essential amino acids as recommended by FAO/WHO [64], for humans, all three products exhibited significantly higher essential amino acids with the exception of Lysine for infants [63]. Solubility characteristics of protein are among the most important functional properties since many functional performances of proteins depend upon their capacity to go into solution [65, 67]. The solubility curve of AC 25, 50 and 60% over a pH range of 2-12 is illustrated in Figure 2. The lowest viscosity was for the 25% AC at 6% hydrolysate concentration (10 Mpa.s) compared to those of 50% AC (50 Mpa.s) and 60% AC (80 Mpa.s) observed at 6 and 7% protein concentrations. All of these factors tend to confound the underlying inverse relationship of protein solubility and viscosity in particular [68].
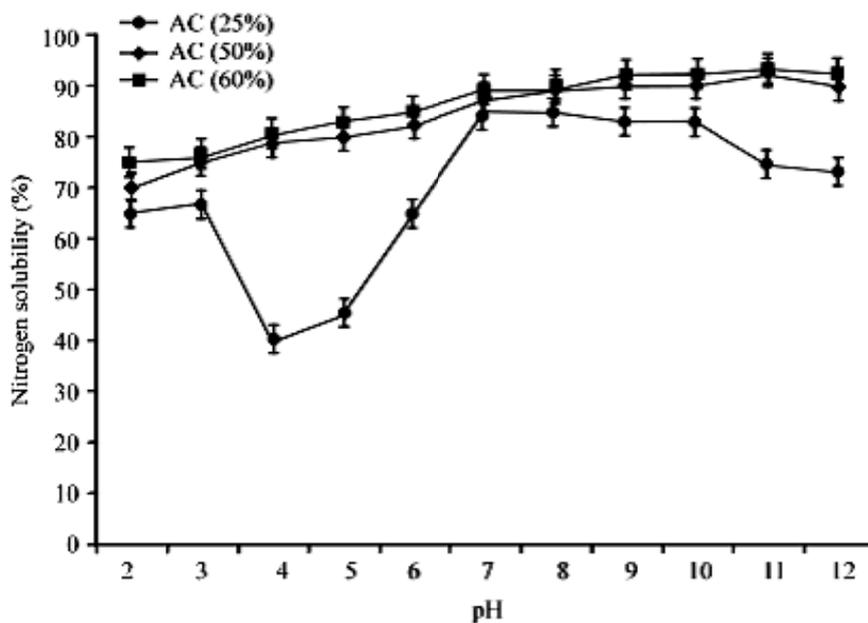
Figure 2. Solubility curve of AC 25, 50 & 60% over a pH range of 2-12 [63].

Preparation of Debittered and Desalted High Fischer Ratio Oligopeptide by Proteolysis of Corn Gluten Meal

The peptide with high levels of branched chain amino acids (BCAA) and low levels of aromatic amino acids (AAA) is called high Fischer ratio oligopeptide. High Fischer ratio oligopeptide is an active peptide with a low molecular weight, derived from various food proteins. High Fischer ratio oligopeptides help to mitigate the symptoms of hepatic encephalopathy [69, 70]. It has been reported that an increase in AAA levels in the brain leads to a decrease in the normal neurotransmitters and an increase in the neurologically inactive phenylethanolamine and octopamine [71], and that BCAA intake improves the plasma amino acid balance. A two step hydrolysis was carried out and ultrafiltration was done [72]. Using ion-exchange method, desalting and debittering was carried out. The comparison of desalinization as affected by different velocities is illustrated in Figure 3.

It is shown that the higher the velocity, the higher the desalted protein recovery ratio and the lower the desalting ratio [72]. The Fischer ratio of oligopeptide (the molar ratio of BCAA to AAA) was 34.71 and the productivity of the high Fischer ratio oligopeptide to the mass of CGM was 11.59%.
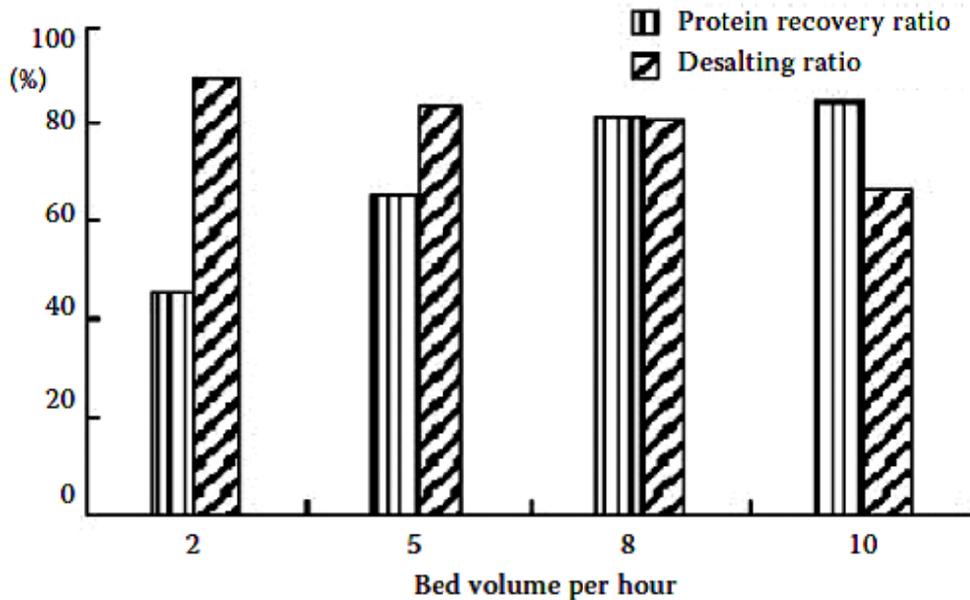
Figure 3. Comparison of desalinization as affected by different velocities [72].

Conclusion

Each method of debittering has its own limitations. Controlled enzymatic debittering using exopepetidases and the subsequent removal of free amino acids serves to be the most economical and effective way to produce hydrolysates. Enzyme inactivation steps should not compromise the integrity of the valuable peptides in the final product. This warrants the need and importance of immobilized enzymes for debittering of the protein hydrolysates.

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