# Enzymatic Hydrolysis of Renewable Vegetable Proteins

# to Amino Acids

Vom Fachbereich Chemie

der Universität Hannover

zur Erlangung des Grades

Doktor der Naturwissenschaften

Dr. rer. nat.

genehmigte Dissertation

von

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Hannover 2002

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Tag der Promotion: 28. Oktober 2002

## Acknowledgements

It is a great pleasure for me to thank Prof. Dr Thomas Scheper who has given me the opportunity to take part on the doctoral program in the Institute of Technical Chemistry, at University of Hannover for the interesting subject and the friendly support.

I wish to express a warm thank to PD Dr. Bernd Hitzmann for acceptance of co-referee and his kindness.

Many friends, colleagues and co-worker helped suggestions and art works for this dissertation. I thank Roland Ulber, Andreas Mackensen, Tanja Koop, Amelita Bartolome, Anke Göhring, Dirk Steuber, Hung Lam, Preyatudsaney Prachaiyo, Wipharat Chuachuad and Jareeya Yimratanabovorn for their help and proof reading.

I'm grateful to Susanna Huebner from OUP (Institut für Ordnungs- und Prozeßpolitik) at University of Hannover for her critical comments and many helpful suggestions in the section of process economics.

I wish to thank Dr. Gregor C. Gams from Gamma Chemie GmbH, who advised me to use enzyme in the way of purpose.

Many measurements of HPLC analysis, I particularly thank Martina Weiß for not only tolerating but embracing this endeavor.

It is a great pleasure for me to thank the colleagues and members who have helped me in the Institute of Technical Chemistry even though we had a matter to communicate and understand to each other.

The financial support of the doctoral program by a grant from the Royal Thai government is gratefully acknowledged.

My grateful thanks to my parents who accepted my absence with great understanding.

Finally, and most importantly, I wish to thank Kusnadi Liem for his constant support, and encouragement, without which this work could not have been completed.

## Abstract

The goal of this work was to study the enzymatic hydrolysis of renewable vegetable proteins. The products can be used for cosmetics, dietetics and pharmaceuticals. Corn gluten meal (CGM) and soybean meal (SBM) were hydrolyzed using the combination of Alcalase 2,4L (an endopeptidase) and Flavourzyme 1000L (an exopeptidase). The effect of enzyme concentration, substrate concentration and reaction conditions on the hydrolysis were investigated. The extent of reaction was expressed in terms of the degree of hydrolysis using reversed-phase HPLC analysis of amino acids. The degree of hydrolysis was also analyzed by *o*-phathaldialdehyde (OPA) and trinitro-benzene-sulfonic acid (TNBS) method, alternatively. These values were then compared using correlation analysis.

The reactive kinetics for the enzymatic hydrolysis were determined. As the product concentration increased, the rate of enzymatic hydrolysis decreased due to the product inhibition. However, the higher the substrate concentration, the more efficient will be the overall process economy. Therefore, substrate concentration should be at the saturation point and the batch reactor should be scale independent in order to provide maximum mixing behavior. Heat treatment of the substrate prior to hydrolysis was essential since the protein denaturation increased the initial rate of hydrolysis according to Linderstrom-Lang's theory.

Alternatively, the reaction using the stable high temperature enzyme, extremozymes such as a recombinant carboxy-terminal protease (from *Thermotoga maritima*) and an isolated protease (from *Thermoanerobacter keratinophilus*) were studied. The characterization of extremozymes, which hydrolyzed different other substrates such as casein and CGM was performed in order to get information about enzyme stability and altered specificity.

The economic costs of the amino acid production process were calculated to serve the purpose of progress control in the available context of the economic evaluation. Beside the economic evaluation and the procedure of enzymatic hydrolysis, ecological advantages were considered.

*Keywords: proteolytic, extremozyme, enzymatic hydrolysis, amino acids*

## Abstrakt

Die Arbeit hatte zum Ziel, die enzymatische Hydrolyse pflanzlicher Proteinen zu untersuchen. Die so gewonnenen Aminosäuren können als Rohstoff für die Kosmetik-, Lebensmittel- und Pharmaprodukte dienen. Maiskleber (CGM) und Sojaprotein (SBM) wurden durch die kombinierte Nutzung der Endopeptidase Alcalase 2,4L und Exopeptidase Flavourzyme 1000L hydrolysiert. Der Einfluß der Enzymkonzentration, der Substratkonzentration und der Reaktionsbedingungen auf die Hydrolyse wurden untersucht. Die Ausbeute der Reaktion wurde durch den Grad der Hydrolyse, welche durch Aminosäureanalyse mit Hilfe der Reversed-phase-HPLC gemessen wurde, dargestellt. Alternativ wurde die Ausbeute der Hydrolyse ebenfalls durch die bekannten *o*-Phathaldialdehyd (OPA)- und Trinitrobenzosulfonsäure (TNBS)-Methoden festgestellt. Diese Werte wurden mit Hilfe der Korrelationsanalyse verglichen.

Die Kinetiken der enzymatischen Hydrolyse wurde bestimmt. Wenn die Produktkonzentration steigt, sinkt die Geschwindigkeit der enzymatischen Hydrolyse aufgrund der Produktinhibierung. Je höher die Substratkonzentration gewählt werden kann, desto wirtschaftlicher ist der gesamte Prozeß. Deshalb sollte die Substratkonzentration am Sättigungspunkt liegen und der Batch-Reactor so ausgelegt werden, dass eine maximale Durchmischung vorliegt. Eine Wärmebehandlung des Substrates vor der Hydrolyse war notwendig, weil die Protein-Denaturierung die Anfangsgeschwindigkeit der enzymatischen Hydrolyse nach der Linderstrom-Lang Theorie verstärkte.

Alternativ könnten Enzyme genutzt werden, die bei hohen Temperaturen stabil sind: Extremozyme. Eine rekombinante carboxy-terminale Protease (aus *Thermotoga maritima*) und eine isolierte Protease (aus *Thermoanerobacter keratinophilus*) wurden eingesetzt. Die Charakterisierung der Extremozyme, die andere Substrate wie Casein und CGM hydrolysieren, wurde durchgeführt, um Informationen über Enzymstabilität und die wechselnde Spezifität zu verbessern.

Die Kosten der Aminosäureproduktion wurden kalkuliert, um eine ökonomischen Auswertung des Prozesses vornehmen zu können. Neben dem Prozeß der enzymatischen Hydrolyse selbst und dessen ökonomischen Berechnung, wurden auch ökologische Faktoren betrachtet.

*Stichwörter: Proteolyse, Extremozyme, enzymatische Hydrolyse, Aminosäuren*

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# 1 Introduction

Commercial interest in amino acids is an outgrowth of an understanding of the various functions that these life-giving substances perform in humans and animals. As understanding of the functions and properties of amino acids increases, new commercial applications arise, while current commercial uses continue to expand their markets on a worldwide basis. Increased knowledge of the role of amino acids in different applications as nutritional agent, led to their use to fortify animal feeds, as food supplements for humans, and to apply them in the pharmaceutical field.

In 1986 the annual production of amino acids in the world was 650,000 tons, with an estimated value of about $2 billion US (Enei et al., 1989). The growth of demand will depend on the progress in production technology to reduce costs, and also on the possible application of amino acids as chemical raw materials (Kaneko et al., 1974). For 1999 the world amino acid demand by region was estimated $5.6 billion US (Figure 1.1). Global demand for amino acids will exceed $7.4 billion US in 2004[1].

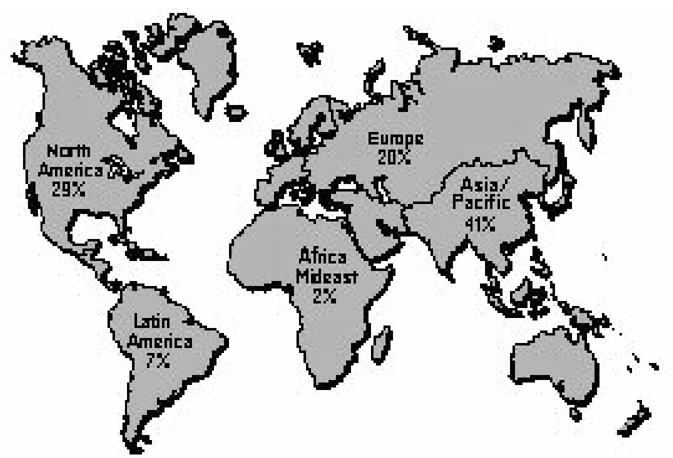


Figure 1.1 Regional demand of amino acid in the world ($5.6 billion US, 1999)[1]

The Asia/Pacific region will continue to be the leader in amino acid production and consumption, largely due to the enormous size of the Monosodium Glutamate (MSG) market in that part of the world, while China's rapid adoption of amino acid feed additives will also increase the demand. However, the saturation of the MSG market will slowly be

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achieved overall in Asia. The most rapid increases will likely take place in the emerging markets of Africa and Latin America (Yoshida et al., 1990).

Thus, the application of amino acids is focused on their nutritional value and taste, and on their physiological activity, involving the food and animal feed industries and the pharmaceutical industry. In practice, this technological excellence emphasize on two areas:

- Technology for improved techniques that enable more efficient production and reduce waste.
- Technology for optimal use of raw materials that can be obtained locally.

The majority of amino acids are produced from vegetable and animal protein sources. The importance of vegetable protein as a protein source in feed has long been recognized. Vegetable protein is economically favorable compared with animal protein such as fish meal, which is source limited. There is no difference in regarding nutritional considerations between amino acid fortification of feed and direct utilization of amino acids for food. However, in the case of food, many complex factors have to be considered, whereas the animal feed production is mainly based on economic factors.

The purpose of amino acid fortification is an increase of animal growth by efficient utilization of vegetable protein, depending on the balance of its constituent amino acids, especially the essential amino acids. Protein from soybean meal is useful, but it is deficient in methionine. All other grains and oilseed meals are primarily deficient in lysine. Lysine and methionine are therefore added to such vegetable based feeds, which result in an increase of animal growth and a reduced amount of excrements (Yoshida et al., 1990).

Soybean meal is a source of vegetable protein that contains a large amount of lysine. Therefore, when soybean meal prices are high and corn prices are low, feed manufacturers tend to use more corn to make cheaper feed. In this event, lysine is added as a nutritional component to the feed. If the price of lysine is high, soybean meal will be added. Today, worldwide about $1.5 \times 10^6$ tons of amino acids are produced annually (Forschungszentrum Jülich GmbH, 2000). While, global demand for lysine is estimated to reach 550,000 tons[2] tannually. The market has grown by an average of 10% annually. For this reason, part of

[2] Estimate for fiscal 2000, Ajinomoto Co., Inc.

the current studies was focused on using vegetable proteins such as soybean meal and corn gluten meal as raw materials in amino acid productions.

Usually, amino acid molecules exist in two mirror image forms, known as the D and L enantiomers, chirals or racemates. L-amino acids are used exclusively for protein synthesis by living organisms. Unfortunately, hydrolysis of proteins in 6 N HCl at 110°C for 24 h inevitably causes racimization of amino acids (Konno, 1993), as obtained 50% L-amino acids and 50% D-amino acids that means, only 50% L-amino acids of the product can be used in living cell. However, some D-amino acids are interesting in food industry, e.g. D-asparagin offers a taste quality of bitterness, and L-asparagin offers a sweetness flavor or a sugar-free sweetener (Enders et al., 1985).

Amino acids are widely prepared by hydrolysis with hydrochloric acid, followed by a neutralization with NaOH. Otherwise, acid hydrolysis lead to some destruction of amino acids and some formal mixture of D- and L-amino acids. The high chemical element and salt concentration arise from neutralization. The separation and purification of amino acids are intended mostly through selective ion-exchange chromatography. In this case, the production of 1 ton of amino acid is accompanied by 0.75 ton NaCl, as a waste that should be considered. Moreover, acid hydrolysis requires high energy for an operation at 120°C, 5 bar for 8 h in an autoclave reactor.

The production of amino acids by acid hydrolysis, according to environmental problems can not be accepted for long term. The aim of environmental protection is to introduce a method which avoids high consumption of chemicals and high salt concentrations, including reduction of energy and waste.

An alternative might be the enzymatic hydrolysis because of milder process condition, higher specificity, easier control of the reaction and minimal formation of by-products (Adler-Nissen, 1985). In addition, the nutritional quality of the amino acids is maintained. The use of thermostable enzymes offers the possibility. As set up an hydrolysis process which works under extreme conditions, for example, high ionic strength in a complex natural substrate. Up to now enzymatic hydrolysis is not in industrial practice for large-scale due to:

- The production costs are too high
- Insufficient enzymatic hydrolysis
- Unsufficient reaction rates

The goal of this project is to study the enzymatic hydrolysis of renewable vegetable protein such as corn gluten meal (CGM) and soybean meal (SBM). Within the process, commercial enzyme and extremozyme are used to carry out the reaction under mild condition, so that the nutritional quality of the amino acid is maintained. The selectivity of these enzymes and raw materials will be studied in detail. The kinetics of the hydrolysis process should be determined in correlation to the established acid process. A detailed economic balance in the purpose of environmental control should be provided in scope of this project.

The final product consists of the residual substrate, enzyme, amino acid and waste water. Further processing, separation and purification of amino acid were studied in other research projects by taking chromatographic polishing and downstream steps.

# 2 Proteins, Amino Acids and Enzymes

## 2.1 Proteins and amino acids

Proteins are polymers of L-$\alpha$-amino acids. The $\alpha$ refers to a carbon with a primary amine, a carboxylic acid, a hydrogen and a variable side-chain group, usually designated as "**R**". Carbon atoms with four different groups are asymmetric and exhibit two different arrangements in space due to the tetrahedral nature of bonds (Barrett., 1985). Amino acids are in either an "L" or "D" configuration, the difference determined by which side of the molecule the amino group (-$NH_2$) is attached. In natural protein, all amino acid residues are of the "L" configuration. Amino acids of the D-configuration are not found in proteins and do not participate in biological reactions (Dawson, 1986). For this reason, D-configured amino acids are not desirable for nutritional supplementation.

Proteins are composed of 20 different amino acids, some of which may be modified after synthesis of the protein. Many amino acids are produced by the body. Others, the essential amino acids, cannot be endogenously synthesized in adequate amounts, so that they must be obtained from the diet. Differences in the properties of amino acids reflect differences among their side chains. Except for glycine, which has no chiral carbon, all amino acids can be classified according to their interactions with water: hydrophobic side chains are derived from compounds that are sparingly soluble in water. Hydrophilic amino acid residues are further subdivided into basic, acidic, or polar non-ionize side chains. The various side-chain groups will influence the chemical properties of proteins as well as determine the overall structure of the protein (Table 2.1 and Table 2.2). The properties of side chains of amino acids are important factors in stabilizing the conformations and determining the functions of proteins (Horton et al., 1993). For example, the polar and non-polar of L-amino acids are:

- ***non-polar*** **:** alanine, glycine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophane, valine.
- ***polar*** **:** arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, histidine, lysine, serine, threonine, tyrosine.

The charges of ionize side chains depend on both the pH and their $pK_a$ values. Differences in charges can be used to separate amino acids and proteins. In proteins, amino acids residues are linked by peptide bonds. The sequence of residues is called the primary structure (see p.7). The formation of peptide bond involves the loss of one molecule of water, OH comes from the carboxyl group and the H from the amino group (Nagodawithana and Reed, 1993).

However, the amino acid composition of a protein can be determined quantitatively by hydrolyzing the peptide bonds and analyzing the hydrolysate (Copeland, 2000).

Table 2.1 Structure of the common amino acids in proteins (Holme, 1998)

| Type of side chain | Name | IUPAC-IUB (Abbreviation) | Structural formula |
|---|---|---|---|
| Carboxylic acid | Aspartic acid | (Ala, A) | HOOC-$CH_2$-$CNH_2$-COOH |
| | Glutamic acid | (Glu, G) | HOOC-$CH_2$-$CH_2$-$CNH_2$-COOH |
| Amide | Asparagine | (Asn, N) | $H_2N$(CO)-$CH_2$-$CNH_2$-COOH |
| | Glutamine | (Gln, Q) | $H_2N$(CO)-$CH_2$-$CH_2$-$CNH_2$-COOH |
| Large hydrophobic side chain | Valine[a] | (Val, V) | $(CH_3)_2$-CH-$CNH_2$-COOH |
| | Leucine[a] | (Leu, L) | $(CH_3)_2$-CH-$CH_2$-$CNH_2$-COOH |
| | Isoleucine[a] | (Ile, I) | $CH_3$-$CH_2$-CH($CH_3$)-$CNH_2$-COOH |
| | Phenylalanine[a] | (Phe, F) | (benzene ring)−$CH_2$−$CNH_2$−COOH |
| | Tyrosine | (Tyr, Y) | HO−(benzene ring)−$CH_2$−$CNH_2$−COOH |
| | Tryptophan[a] | (Trp, W) | HC, CH, C, HC, CH, N, NH, C−$CNH_2$− COOH, CH (ring structure) |
| | Proline | (Pro, P) | $H_2C$ − $CH_2$, $H_2C$, CH − COOH, NH (ring structure) |
| | Hydroxyproline[b] | (Hyp) | HOHC−$CH_2$, $H_2C$, CH−COOH, NH (ring structure) |
| S-Containing side chain | Cysteine | (Cys, C) | $HSCH_2$-$CNH_2$-COOH |
| | Methionine[a] | (Met, M) | $CH_2$-S-$CH_2$-$CNH_2$-COOH |
| Small neutral side chain | Lysine[a] | (Lys, K) | $H_2N$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CNH_2$-COOH |
| | Arginine | (Arg, R) | $H_2N$-(CNH)-NH-$CH_2$-$CH_2$-$CH_2$-$CNH_2$-COOH |
| | Histidine | (His, H) | N−CH, HC, CH−$CH_2$−$CNH_2$− COOH, NH (ring structure) |
| Basic side chain | Glycine | (Gly, G) | H-$CNH_2$-COOH |
| | Alanine | (Ala, A) | $CH_3$-$CNH_2$-COOH |
| | Serine | (Ser, S) | $HOCH_2$-$CNH_2$-COOH |
| | Threonine[a] | (Thr, T) | $CH_3$-CHOH-$CNH_2$-COOH |

[a] Essential nutrition to humans

[b] Occurs in gelatin, which is produced from collagen

Table 2.2 Some characteristics and properties of the common amino acids in protein

| Name | Molecular weight (MW) | Residue molecular weight (MW)[b] | Nitrogen content (%) | Water solubility (g/100g, 25°C)[c] | M.p (°C) | pI at 25°C $\left(\frac{pK_1 + pK_2}{2}\right)^d$ |
|---|---|---|---|---|---|---|
| Aspartic acid | 133.1 | 115 | 10.52 | 0.78 | 270 | 2.98 |
| Glutamic acid | 147.1 | 129 | 9.52 | 0.85 | 249 | 3.08 |
| Asparagine | 132.1 | 114 | 21.21 | 3.53 | 236 | 5.40 |
| Glutamine | 146.1 | 128 | 19.17 | 3.60 | 185 | 5.70 |
| Valine | 117.2 | 99 | 11.96 | 8.85 | 315 | 6.00 |
| Leucine | 131.2 | 113 | 10.68 | 2.43 | 337 | 6.04 |
| Isoleucine | 131.2 | 113 | 10.68 | 4.12 | 284 | 6.04 |
| Phenylalanine | 165.2 | 147 | 8.48 | 2.97 | 284 | 5.91 |
| Tyrosine | 181.2 | 163 | 7.73 | 0.05 | 253 | 5.63 |
| Tryptophan | 204.2 | 186 | 13.72 | 1.14 | 282 | 5.88 |
| Poline | 115.1 | 97 | 12.17 | 162.3 | 222 | 6.30 |
| Hydroxyproline | 133.1 | 113 | 10.68 | -- | -- | 5.80 |
| Cysteine | 120.1[a] | 103 | 11.57 | v.sol | 178 | 5.02 |
| Methionine | 149.2 | 131 | 9.39 | 3.38 | 283 | 5.74 |
| Lysine | 146.2 | 128 | 19.17 | v.sol | 224 | 9.47 |
| Arginine | 174.2 | 156 | 32.18 | 15 | 238 | 10.76 |
| Histidine | 155.2 | 137 | 27.09 | 4.19 | 277 | 7.64 |
| Glycine | 75.1 | 57 | 18.66 | 25 | 292 | 6.06 |
| Alanine | 89.1 | 71 | 15.73 | 16.65 | 297 | 6.11 |
| Serine | 105.1 | 87 | 13.33 | 5.02 | 228 | 5.68 |
| Threonine | 119.1 | 101 | 11.76 | 20.5 | 253 | 6.5 |

[a] MW of cystine is 2 × 120.1

[b] Residue molecular weight is the MW minus water or 18.

[c] Food proteins: properties and characterization, Nakai, S. and Modler, W.H. (1996).

[d] The Merck Index, Merck & Co. Inc., Nahway, N.J., 11(1989); CRC Handbook of Chem.& Phys., Cleveland, Ohio, 58(1977).

### 2.1.1 Types of protein structure

The protein-protease interaction is related to its structure. The following levels of organization should be considered when describing the structure of a protein.

***Primary Structure*:** The sequence of the amino acids that makes up the protein is different for every protein. The overall protein structure and function are determined by the sequence

of amino acids in the protein. The number of peptide bonds that a protease can break, depend on which amino acids are in the protein and which amino acids are adjacent to each other.

***Secondary Structure*:** Depending on which amino acids are present, the peptides chain can organize itself in several ways. For example, it can coil up to form an "α-helices" similar to a bedspring. In some cases the peptide chain can be in an extended zigzag state referred to as the "β-conformation". Finally it may take neither form, existing as a "random coil".

***Tertiary Structure*:** The tertiary structure describes the shape in which the protein chain is folded its three dimensional (3D) conformation. This is influenced by the areas of α-helices and random coils that are in the protein chain. The tertiary structure is greatly affected by intra chain forces and very important in determining how susceptible a particular protein is to attack.

***Quaternary Structure*:** This refers to the association of several proteins to form an aggregate. For example, several 30,000 MW soy protein sub-units associate to form larger complexes of about 300,000 MW units. The forces that cause sub-units to associate are similar to those that stabilize the tertiary structure. It may be necessary to breakup the protein aggregates in order to achieve extensive hydrolysis.

### 2.1.2 Vegetable protein sources

Unlike animal proteins, plant proteins may not contain all the essential amino acids in the necessary proportions. Protein sources from oilseed such as soybean meal are useful but it is deficient in methionine. The limiting amino acid in cereals is lysine, without exception.

Vegetable proteins are a prominent by-product emerging from the processing of starch and oil, for example, corn gluten meal and soybean meal which were used as substrates in this work.

#### 2.1.2.1 Con gluten meal (CGM)

There are three basic methods for processing corn: alkaline processing, dry milling and wet milling is a process that utilizes mechanical unit operations size reduction (milling), aqueous extraction and separation (filtration, centrifugation) for separating and purifying the major corn components, viz., oil, starch, protein, fiber. However, wet milling generates large amounts of protein co-products: about 5-6% of the corn becomes corn gluten meal (CGM, 60% protein) and 24% of corn dry weight is corn gluten feed (CGF, 21% protein).

Figure 2.1 is a flow diagram of the process steps in wet milling corn. The $SO_2$ in the steeping step not only helps disrupt disulfide bonds and protein matrix, but it also helps activate proteases and provides a favorable pH for a lactic acid fermentation. Protein-containing products of wet processing are: a) the soluble (also called steep water, steep liquor or fermented corn extractives); b) the germ residue remaining after oil extraction; and c) the gluten, which is the insoluble proteinaceous portion of the endosperm. The dry substance in steep water analyses about 50% crude protein (N × 6.25), but only about 14% of this crude protein is non-dialyzable. Steep water is a good source of amino acids and growth factors desired as a supplement to many industrial fermentation (Christianson et al., 1965). Of these three protein source, corn gluten possesses more possibilities as a raw material for industrial applications because of its high protein concentration, usually about 68% dry basis After wet milling, nearly 45% of the protein in corn is found in gluten constituting 60% of that protein.

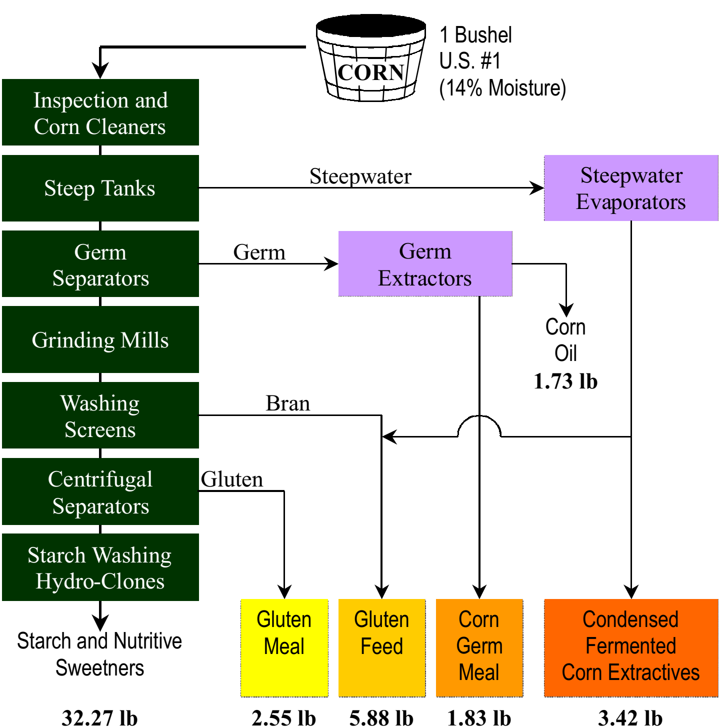



Figure 2.1 Corn wet-milling process flow diagram (1 bushel = 25 kg, 1 lb = 0.454 kg).

### 2.1.2.2 Soybean meal (SBM)

SBM is a processed by-product of soybeans crushing industry that produces vegetable oil for human consumption. This 44-48% protein meal is the most common source of protein in feed and is commonly used in poultry, hog and dairy rations. Solvent extracted soybean meal is produced by cracking, heating and flaking soybeans and reducing the oil content of the

conditioned product by the use of hexane or homologous hydrocarbon solvents to 1% or less on a commercial basis. The extracted flakes are cooked and ground into meal. Soybean meal of 49-50% protein with a maximum of 3% fiber is produced in areas where the protein content is higher in the original soybean. It may contain an inert non-toxic conditioning agent, either nutritive, or non-nutritive, or a combination therefore, to reduce caking and improve flow ability to accomplish its intended effect. The name of conditioning agent must be shown as an added ingredient on the label that are determined by the Association of American Feed Control Officials Incorporated (AAFCO, 2001).

### 2.1.3 Amino acid properties

An amino acid is any molecule that conforms, at neutral pH, to the general formal:

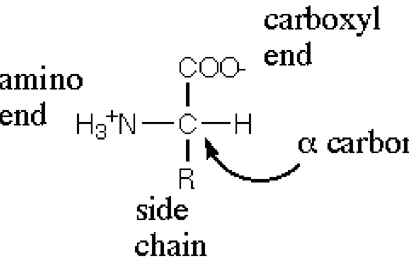



Figure 2.2 General structure of an α-amino acid.

The central carbon atom in this structure is referred to as the *alpha carbon* ($C_\alpha$) because it is adjacent to the carboxy (acidic) group. Each amino acid has two chemical groups, an amino group and a carboxyl group, which can form chemical bonds with other amino acids. An R group, is known as the *amino acid side chain.* Amino acids are classified into the following groups based on the chemical and/or structural properties of their side chains (Figure 2.2).

Table 2.3 Classification of amino acids based on chemical structure (David and Peck, 1998).

| Chemical nature of R group | Examples |
|---|---|
| Aliphatic | Gly, Ala, Val, Leu |
| Aromatic | Phe, Tyr, Trp |
| Hydroxylic | Ser, Thr |
| Carboxylic | Asp, Glu |
| Sulphur containing | Cys, Met |
| Imino | Pro, Hyp |
| Amino | Lys, Arg |
| Amide | Asn, Gln |

In the simplest case, R=H (amino acetic acid or glycine). In other amino acids R is an aliphatic, aromatic or heterocyclic residue and may incorporate other functional groups. Since the alpha carbon is a chiral center, all the naturally occurring amino acids, except glycine, exist in two enantiomeric from the L and D. All naturally occurring proteins are composed exclusively from the L enantiomers of amino acids.

It is helpful when considering the principles and applications of methods for the determination of amino acids to be able to appreciate the characteristics of these compounds (Table 2.3). Although it is not always essential to know the exact structural formula of individual amino acids it is useful to remember particular properties or the presence of functional groups (Rex et al., 1995).

#### 2.1.3.1 Isomerism

Because of alpha carbon of amino acids is an asymmetric carbon, each amino acid molecules can exist in two mirror image forms, known as L- and D-enantiomers, chirals or racemats (Figure 2.3). The amino acids present in living system are almost exclusively L-isomers. The exceptions are a few D-amino acids present in the antibiotics produced by fungie.

H
$CH_3-C-COOH$ ═ $CH_3-C-COOH$ (with $NH_2$ above and H below the second C)
$NH_2$
L-Alanine D-Alanine

Figure 2.3 The stereoisomers of L and D-alanine.

The amino acids which occur in proteins are in the L-form, and the final form of amino acids utilized in the living body is also of the L-configuration. Although it is well known that certain D-amino acids are nutritionally available, they are utilized only after being transformed to the L-form. Therefore, a mixture of L-amino acids is considered to have greater availability than a mixture of DL-amino acids

In fact, Howe et al. (1946) demonstrated that a decrease in the use of racemic amino acids and an increase in the use of the corresponding L-antipodes improved both the tolerance to and effectiveness of amino acid infusion. Based on these facts, and also as a result of the recent progress in amino acid production, mixtures containing only L-form amino acids have come into wider use for parental infusion.

### 2.1.4 The production of amino acids

Industrialization of amino acid production was initiated in 1909. The first step was the production and use of monosodium L-glutamate as a tasty substance. It has been produced by extraction from protein hydrolyzates (Kaneko et al., 1974), but this method has the drawback of yielding a large volume of by-products. In the technological revolution, new methods of production were sought and in 1956 a fermentation method was introduced. The fermentation methods for the production of most amino acids are now available (Yoshida et al., 1990). Methods of synthesis for glutamic acid and methionine were industrialized in 1960. Even though the chemical synthesis of glutamic acid requires an optical resolution step to obtain the L-isomer, this method remains competitive with the fermentation processes. In the case of methionine, L-methionine and D-methionine are of equal nutritive value (Jackson and Rose, 1938). Therefore, the complicated process of resolution of the two isomers can be omitted, and DL-methionine is supplied directly for practical use. DL-Methionine is utilized in the body equally to L-methionine, since D-methionine is deaminated to the corresponding α-keto acid, which produces L-methionine by transamination (Stryer, 1995). Thus, the synthetic method of methionine is particularly advantageous in this case. Although methods of chemical synthesis have been studied intensively, the fermentation method remains the cheapest. Most amino acids are now produced by chemical synthesis, fermentation, and extraction (Yoshida et al., 1990).

Alternatively, biosynthetic production, enantiopure L- and D-amino acids were synthesized by enzymatic synthesis. Enzymatic hydrolysis produced amino acids as defined in the pharmaceutical quality these require the optical purity of L-amino acids and avoid inorganic compounds contaminate the amino acids preparation during the process (National formulary, 1970; The pharmacopoeia of Japan, 1973).

### 2.1.5 The application of amino acids

Amino acids are constituents of proteins, and essential nutrients for living organisms. Demand for amino acids is therefore based on such areas as medicines, food additives and additives for animal feed. The potential demand for amino acids as food and feed additives is enormous on relation to their nutritional value and taste. In medical fields is expected to broaden further as the specific physiological functions of individual amino acids become clearer based on new research into protein and amino acid metabolism in various diseases.

Application of the characteristics of amino acids by combining them into cosmetic preparations has been widely attempted in recent years. However, the amino acids and their derivatives examined in use of cosmetics is limited, and their application in this field seems very promising. The cosmetic markets with amino acids based products that ingredients have been favorably evaluated as mild to the skin, extremely safe and highly biodegradable, thus, these data defined the importance and development of the production of amino acids to economy and ecology. The growth demand will therefore depend on progress in production technology to reduce costs, and also on the possible application of amino acids. The areas of application of amino acids are the following:

I Pharma/health food/cosmetic (e.g. Tryptophane, Leucine, Isoleucine, Valine); 20%

II Animal feed (e.g. Methionine, Lysine, Threonine); 30%

III Food (e.g. Glutamic acid, Phenylalanine); 50%

## 2.2 Proteases

### 2.2.1 Classification of proteases

The International Union of Pure and Applied Chemistry (IUPAC) formed the Enzyme Commission (EC) to develop a systematic numerical nomenclature for enzymes. The EC classifications are based on the reactions that enzymes catalyze. Six general categories, have been defined, as summarized in Table 2.4. Within each of these broad categories, the enzymes are further differentiated by a second number that more specifically defines the substrates on which they act.

Table 2.4 The IUPAC EC classification of enzymes into six general categories according to the reaction they catalyze

| First EC number | Enzyme class | Reaction |
|---|---|---|
| 1 | Oxidoreductases | Oxidation-reduction |
| 2 | Transferases | Chemical group transfers |
| 3 | Hydrolases | Hydrolytic bond cleavages |
| 4 | Layses | The cleaving of bonds by reactions other than hydrolysis. |
| 5 | Isomerases | Changes in arrangements of atoms in molecules |
| 6 | Ligases | The formation of bonds and require ATP |

Each enzyme has been given a four-digit number by the Enzyme Commission of the International Union of Biochemistry. The first three digits relate to the reaction catalyzed by the enzyme and the final one is required if several enzyme with different protein structures catalyze the same reaction.

**Name of enzyme (EC W.X.Y.Z)**

**EC** - Enzyme Commission number system
**W** - indicates the reaction catalyzed (1-6)
**X** - indicates the general substrate or group involved
**Y** - indicates the specific substrate or coenzyme
**Z** - the serial number of the enzyme

Table 2.5 Types of peptidases defined in the Enzyme Nomenclature list of the International Union of Biochemistry and Molecular Biology (1992).

| EC number | Peptidase type | Action |
|---|---|---|
| Exopeptidases | | |
| 3.4.11.- | Aminopeptidase | N-terminal residue released |
| 3.4.13.- | Dipeptidase | Acts only on dipeptides |
| 3.4.14.- | Dipeptidyl peptidase | N-terminal dipeptide released |
| | Tripeptidyl peptidase | N-terminal tripeptide released |
| 3.4.15.- | Peptidyl dipeptidase | C-terminal dipeptide released |
| 3.4.16.- | Carboxypeptidase (serine) | C-terminal residue released |
| 3.4.17.- | Carboxypeptidase (metallo) | C-terminal residue released |
| 3.4.18.- | Carboxypeptidase (cysteine) | C-terminal residue released |
| 3.4.19.- | Omega peptidase | Releases modified residues from N- or C-termini |
| Endopeptidases | | |
| 3.4.21.- | Serine endopeptidase | |
| 3.4.22.- | Cysteine endopeptidase | |
| 3.4.23.- | Aspartic endopeptidase | |
| 3.4.24.- | Metallo endopeptidase | |
| 3.4.99.- | Endopeptidase of unknown catalytic mechanism | |

Proteases are classified according to their source (animal, plant, microbial), their catalytic action (endopeptidase or exopeptidase), and the nature of the catalytic site. In the EC system for enzyme nomenclature, all proteases (or peptide hydrolyases) are in subclass 3.4, which is further divided into 3.4.11-19, the exopeptidases, and 3.4.21-24, the endopeptidases or proteinases. Endopeptidases are the proteases most commonly used in food processing, but in some cases their action is supplemented with exopeptidases.

Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, whereas exopeptidases hydrolyze one amino acid (or dipeptide, in the case of 3.4.14 and 15) at a time from either the N terminus (aminopeptidases) or the C terminus (carboxypeptidases) (Figure 2.4). These types of endo and exopeptidases illustrated in Table 2.5 (Sterchi and Stöcker, 1999).

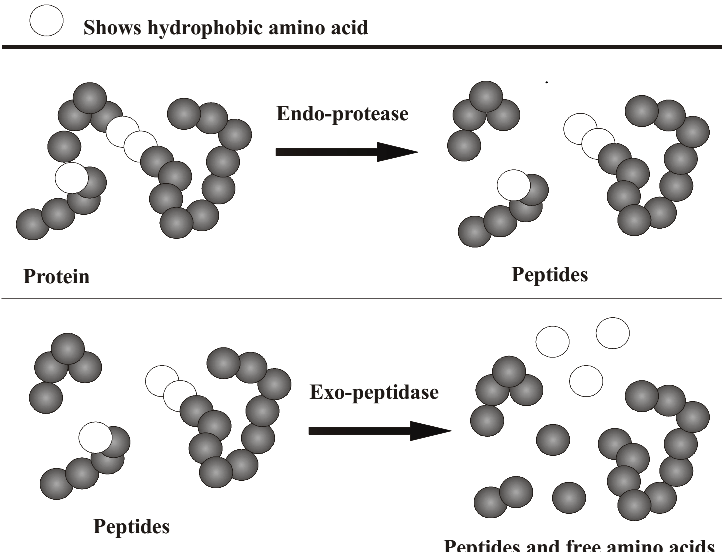



Figure 2.4 Mode of action of the different type of proteases hydrolyzing different bonds in a protein.

### 2.2.2 Endopeptidases

The four major classes of endopeptidases are serine proteases (EC 3.4.21), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23) and metallo proteases (EC 3.4.24).

The serine proteases have maximum activity at alkaline pH; the closely related cysteine proteases usually show maximum activity at more neutral pH values. The aspartic proteases generally have maximum catalytic activity at acidic pH (Beyon and Bond, 1989).

The metallo proteases contain an essential metal atom, usually Zn, and have optimum activity near neutral pH. $Ca^{2+}$ stabilises these enzyme and strong chelating agents, such as EDTA, inhibit them. Such enzymes are common in microorganism.

### 2.2.3 Exopeptidases

The aminopeptidases (EC 3.4.11) are ubiquitous, but less readily available as commercial products, since many of them are intracellular or membrane bound. The commercial enzyme preparation Flavourzyme®, isolated from *Aspergillus oryzae*, contains both endoprotease plus aminopeptidase and carboxypeptidase activities. Flavourzyme can be used for extensive hydrolysis of proteins resulting in taste development (Novo Nordisk).

Carboxypeptidases are subdivided into serine carboxypeptidases (EC 3.4.16), metallo carboxypeptidases (EC 3.4.17) and cysteine carboxypeptidases (EC 3.4.18) according to the nature of the catalytic site. Many commercial proteases, in particular from fungi, contain appreciable amounts of carboxypeptidases activity.

Evolutionary classification of peptidase based on amino acid sequences (from Barrett et al., 1998 and MEROPS - the peptidase database
url:http://www.bi.bbsrc.ac.uk/Merops/merops.htm)

### 2.2.4 Safety and regulatory aspects of the use of enzymes

Enzymes are used in different ways in the processing of food, and their legal status depends on the application. In the former case, the enzyme might be considered as an additive and subjected to the statutory additive safety testing program.

***Purity and food-grade specifications*:** The commercial enzyme preparation should conform at a minimum to the *Food Chemicals Codex* specifications for enzyme preparations. Impurities specific to the source of the enzyme or to the manufacturing process should also be identified and measured. A limit upon such impurities be required, it will be incorporated into the food-grade specifications. The source of enzyme has given some cause for concern as toxins may be incorporated into the crude enzyme preparations. The minimum testing requirement have been defined together with guidelines for good manufacturing practice.

***Intended use and use levels*:** Enzymes are food additives in which an enzyme preparation is intended to be used should be specified. Enzyme preparations always contain other

substances (salts, preservatives, stabilizer, carriers, nonenzymic organic material, etc.) Further, some enzymes are added to food and remain there, although they may be inactivated by heat or other treatment in the finish food product. On the other hand, some enzymes only come in contact with the food (immobilized enzymes) but do not stay there. For these reasons, it is not an easy matter to estimate total enzyme use and consumption. This is necessary to allow calculation of probable human consumption of the enzyme preparation. If the enzyme preparation is directly added to food, its use level, or range of levels, should be provided for each food group and expressed as milligrams of total organic solids (TOS)[3] per gram or kilogram of food.

Safety evaluation should focus on possible contaminants which could be present. At present, there is no evidence to link consumption of added enzymes in food with any deleterious effects in humans (Pariza and Foster, 1983; Beutler, 1984; Food Chemicals Codex, 1981).

[3] TOS (%) = 100 - A - W – D
where; A = % ash, W = % water, D = % diluents or carriers if enzyme is immobilized

# 3 Analyses

## 3.1 Analytical nitrogen contents

Nitrogen in protein of corn gluten meal (CGM) and soybean meal (SBM) does not only come from amino acids in protein, but also exits in additional forms that may or may not be used as a part of the total nitrogen economy of humans and animals. In addition, purines, pyrimidines, free amino acids, vitamins, creatine, creatimine and amino sugars can all contribute to the total nitrogen present. It is therefore ideal to use analytical nitrogen which measures the total nitrogen content lead to calculate their amount of crude protein. One such method which most commonly used is the Kjeldahl method.

In practice, most biological methods for evaluating protein quality are, in fact, evaluating nitrogen but are expressed as crude protein (N × 6.25) (FAO/WHO, 1973). The calculation of "crude protein" by multiplying total nitrogen by 6.25 (see Annex 9.3) can give a serious overestimation of protein content. The protein content of foodstuffs is conventionally estimated from the nitrogen content determined by the Kjeldahl technique. Numerous modifications of the original procedure have been proposed (Munro, 1969). A recommended method based on the procedure of the Association of Official Analytical Chemists (AOAC, 1975). In this method, the standard conversion factor, which should be used to calculate the amount of crude protein contents, is 6.25. However, the absolute size of the factor for various proteins has been much debated, but in practice this problem is rarely critical and can be disregarded (Nagodawithana and Reed, 1993).

From a nutritional perspective, it is evidently important to know the amount of crude protein in raw materials, because this data is also a necessary information for the calculation of total hydrolysis ($h_{tot}$). $h_{tot}$ is the number of peptide bonds per gram protein and it has units of mmol amino acid per gram crude protein, $N \times f_N$. The number of peptide bonds is calculated from the amino acid composition as the sum of the concentrations of each amino acid. These amino acid concentrations are obtained immediately from the amino acid analysis, in which the concentrations usually are given as mg or g amino acids per liter. Thus, $h_{tot}$ is used to calculate the degree of hydrolysis (DH) (see Annex 9.8). It is defined as the percentage of peptide bonds cleaved and is a key parameter which characterizes a protein hydrolysate:

$$\% \text{ DH} = \frac{\text{Number of peptide bonds cleaved}}{\text{Total number of peptide bonds}} \times 100\%$$

In this work, the crude proteins of corn gluten meal (CGM) and soybean meal (SBM) (see 2.1.2), were supplied by Amino GmbH, Braunschweig (Figure 3.1). We send these vegetable proteins to various laboratories for measuring nitrogen content by the Kjeldahl method (see Annex 9.4). The average on precise value would be used on the hydrolysis experiment further. As crude protein of CGM and SBM were 63% and 50%, respectively (Table 3.1).

Figure 3.1 Vegetable proteins as substrate: *left side* is corn gluten meal (CGM) and *right side* is soybean meal (SBM), were supplied by Amino GmbH, Braunschweig.

Table 3.1 Micro-Kjeldahl *N* method done by various laboratory

| **Source** | **Kjeldahl N(%)** | | **Protein (%) [N $\times f_N$[1]]** | |
|---|---|---|---|---|
| | **corn gluten meal** | **soybean meal** | **corn gluten meal** | **soybean meal** |
| Amino GmbH | 10.10 | 8.20 | 63.13 | 51.25 |
| SIWA Lab | 9.94 | 7.78 | 62.13 | 48.63 |
| Göttingen Uni | 10.05 | 8.02 | 62.82 | 50.15 |
| **average value** | **10.03** | **8.00** | **62.69** | **50.01** |

[1] $f_N$ = 6.25 (conversion factors)

## 3.2 Amino acid analysis

A liquid chromatographic approach to automatic amino acids analysis dominated by "Moore and Stein technique" was adapted to complex, costly and inflexible instrumentation (Spackman et al., 1958). Since there are a number of methodologies available for the high performance liquid chromatography (HPLC) analysis of amino acids. The best method for the special application can be chosen. When rapid, high sensitivity analysis of only primary amino acids is required, pre-column derivatization with *o*-phthalaldehyde (OPA) should be considered (Pfeifer et al., 1983). It is a method still in its infancy, but its advantages are appealing. For amino acid screening or analysis of selected amino acids in a mixture, it can prove to be an ideal method.

In this work, amino acids analysis was done mainly using reverse phase-HPLC columns and pre-column derivatization with OPA reagent. Buffered mobile phases were used. The proportions of polar solvents (e.g. methanol, tetrahydrofuran) depend on the type of the derivative employed. Herein, derivatization with OPA was used because it is simple and the poor reproducibility due to the instability of the reaction product can be improved by automation of the procedure and substitution of ethanethiol with the 2-mercaptoethanol in the derivatizing reagent. Gradient elution was required for the resolution of complex mixtures and the overall analysis times was 67 min (Figure 3.4).

### 3.2.1 Amino acid analysis using reversed -phase HPLC

The analysis and purification of biological molecules using revered-phase (hydrophobic) HPLC is a widely employed technique. It is useful for the separation of a wide variety of water and alcohol soluble compounds (Pfeifer et al., 1983). The use of RP-HPLC was applied to amino acid analysis.

The most commonly employed technique for RP-HPLC analysis of amino acids is pre-column derivatization. With this method, the ∝-amino group of each amino acid is reacted with a hydrophobic derivatizing agent to yield a readily chromatographic product. With the proper derivatizing agents, each amino acid will form a single and unique product. The method of detection will be determined by the properties of the reagent, with sensitivity and specificity being two primary detection criteria (Klapper, 1982). Ideally, pre-column derivatives for amino acid analysis will either fluorescence or absorb light in the region above 300 nm to prevent detection of sample. Sensitivity levels should be in the low picomole range. The HPLC instrument of amino acids analysis was used in this experiment, is shown in Figure 3.2.

### 3.2.2 Automated pre-column derivatization with OPA

Ortho-phthalaldehyde (OPA) was required for a pre-column derivatization agent for primary amino acids (Hill, et al., 1979). Each primary amino acid forms a single, unique, substituted isoindole product that can be detected with fluorescence at sub-picomole levels. The relative instability of the derivatives makes the OPA reaction a perfect test for the technique of automated pre-column derivatization.

For these measurements, a sample vial containing the OPA solution (5 mg OPA/ml borate buffer) was placed in position 1 of the automatic sample processor. The samples and standards were placed in the remaining positions. Upon initiation of an analysis run, 5µl of the OPA solution was withdrawn followed by 5 µl of sample (or standard). After mixing, the amino acids were allowed to react with the OPA for 1.5 min. Immediately following reaction, the derivatized products were applied to and separated on a Resolve™ 5 µm $C_{18}$ column (Annex 9.3). A schematic for this process is shown in Figure 3.3. It is possible to perform automated analysis of even unstable amino acid derivatives with good accuracy and reproducibility. The effect of signal on typical peaks of amino acids analysis of corn gluten meal hydrolysate using this process is shown in Figure 3.4, for example. The advantages of pre-column derivatization with OPA become immediately apparent. Reaction of the primary amino acids, including glutamine and asparagine, was excellent. By overcoming the problem of derivative instability, routine automated analysis using pre-column derivatization with OPA is now feasible.

Figure 3.2 Instrument of amino acid analysis, HLPC; RF 10AXL fluorescence detector, Shimadzu; Resolve™ 5 µm $C_{18}$ , 4 × 150 mm, Reversed phase Waters.

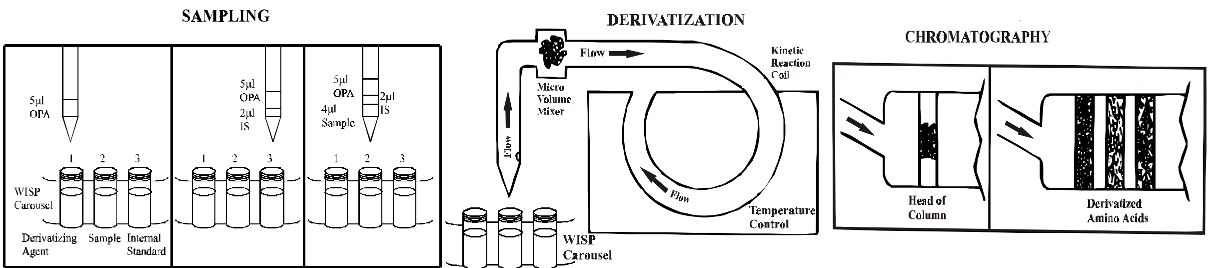



Figure 3.3 Automatic pre-column derivatization procedure. The sample processor is programmed via the system controller to meter derivatization reagent and sample into the sample loop. These solutions are automatically mixed and the reaction is precisely timed. The derivatized sample components are then automatically injected and enriched at the head of the column. Components are separated by high resolution gradient elution and flow programmed chromatography.

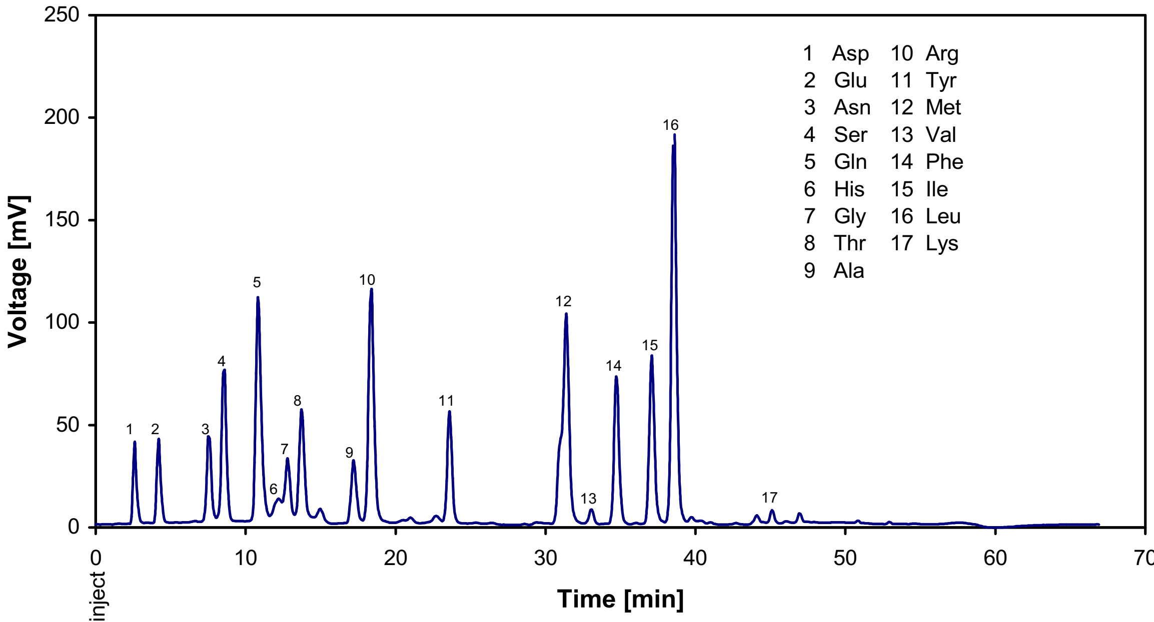



Figure 3.4 Rapid, high sensitivity analysis of primary amino acids of corn gluten meal hydrolysate using automated pre-column derivatization with OPA. Solvent A: 0.05 M $Na_2HPO_4$, pH 7: $CH_3OH$ : THF, 96:2:2; solvent B: $CH_3OH$ : $H_2O$, 65:35; 1 ml/min, Sample: 50 pmol each amino acid. Detection: Waters model RF 10AXL fluorescence detector, ex: 330 nm, em: 420 nm, gain: 8x. Column: Resolve 5 µ $C_{18}$ column (15 cm × 4 mm).

## 3.3 Alternative method for determining degree of hydrolysis

### 3.3.1 Introduction

HPLC (high performance liquid chromatography) using reversed phase system has become popular for the separation of amino acids. The excellent separation performance, however, makes it useful for amino acids analysis of enzymatic protein hydrolysate. The success of automated amino acid analysis, on complicated and very expensive instruments, is often determined by the performance of an HPLC column. Moreover, the period of analysis is time-consuming about 67 min of the injection, as it can not monitoring during hydrolysis.

In protein hydrolysis, the key parameter for monitoring the reaction is the degree of hydrolysis (DH) see Annex 9.8. The DH determination should be based on a selective and appropriately sensitive and rapid analytical method. The extent of proteolysis can be determined by a suitable end group assay, and here the analysis of free amino groups by trinitrobenzenesulphonic acid (TNBS) has become the most widely applied. A version of the TNBS method which is particularly suitable for analyzing hydrolysates together with insoluble protein has been developed by Adler-Nissen (1979). However, this method is not completely selective for primary amino acid groups because of color development due to hydroxy-ions present in the medium (Adler-Nissen, 1979). The method does have its drawbacks. As it is not possible to obtain results quickly enough during hydrolysis to follow the process closely. In addition, the TNBS reagent is unstable, toxic, and has to be handled carefully due to the risk of explosion. Furthermore, TNBS cannot be applied if mercaptoethanol has been used to disrupt disulphide bonds in the protein molecule, because the reaction products also absorb at the measured wavelength of 340 nm (Kotaki et al., 1964).

Alternatively, *o*-phthaldialdehyde (OPA) (Church et al., 1983), which is more selective for α-amino groups than TNBS. This reagent may be used to react only with amino groups or its derivatives. The nitrogen atom is the essential factor for color development in this color reaction. However, to provide a suitable method, a reaction was selected between amino groups and *o*-phthaldialdehyde (OPA) in presence of β-mercaptoethanol forming a colored compound detectable at 340 nm in a spectrophotometer.

In this work, the OPA and TNBS method were compared to establish was accurate method to determine the degree of hydrolysis. With the OPA method, dithiothreitol (DTT) was used instead of β-mercaptoethanol (see Annex 9.10) because this reagent was environmentally more acceptable. Moreover, it was stable and less toxic than the TNBS reagent. The correlation of the OPA method with the widely accepted TNBS method (Annex 9.9) were

investigated, especially in relation to protein hydrolyses with degree of hydrolysis and using CGM and SBM as substrates.

### 3.3.2 Correlation analysis between TNBS and OPA method

CGM and SBM were suspended in deionized water with a protein concentration of 8% protein (w/w). The pH was adjusted to 9.0 with 1 N NaOH before adding the enzyme and no pH adjustment during hydrolysis. Alcalase 2,4 L and Flavourzyme 1000 L type FG (Novo Nordisk A/S, Bagsvaerd, Denmark) were added in the suspension, dosages of 2% and 2.5% respectively, based on weight protein percent. During hydrolysis pH drops typically to about pH 6. Following enzyme addition, samples were taken after 2, 4, 6, 8, 10, 12, 16, 18, 22 and 24 h, heated at 85°C for 5-10 min to inactivate the enzymes and frozen for later analysis.

The degree of hydrolysis (DH) of CGM and SBM by OPA and TNBS methods are illustrated in Figure 3.5, Figure 3.6 and Table 3.5 with HPLC analysis as their reference. The average and standard deviation (SD) of both methods were determined by 10 replicates for each sample. The results are shown in Table 3.2 and Table 3.3 for CGM and SBM respectively. These data illustrate that the OPA method was more reactive toward the α-amino groups of SBM than CGM. Because of the difference in the availability of the α-amino groups in CGM and SBM, the content of lysine of SBM is higher than CGM (Adler-Nissen, 1986). The availability of the α-amino groups can be reduced significantly by Maillard reactions (reaction between simple sugars and amino acids and simple peptides at room temperature that cause browning) in the proteins (Monner et al., 1992). Thus, there were options of reaction with the α-amino groups (lysine) and the amino groups at the position of the protein molecule led to background color from the samples which might interfere with the color formed by the reagent.

Thus, the OPA and TNBS methods should be used with caution at very low DH (Nielson et al., 2001). At very high DH of CGM in Figure 3.5 that the overall reaction curves of both methods seem different from the reference HPLC analysis. However, Figure 3.6 illustrates the initial velocity of DH of SBM at very low DH; the curves of both methods were not different from the reference, but the DHs determined by both methods were different from the reference.

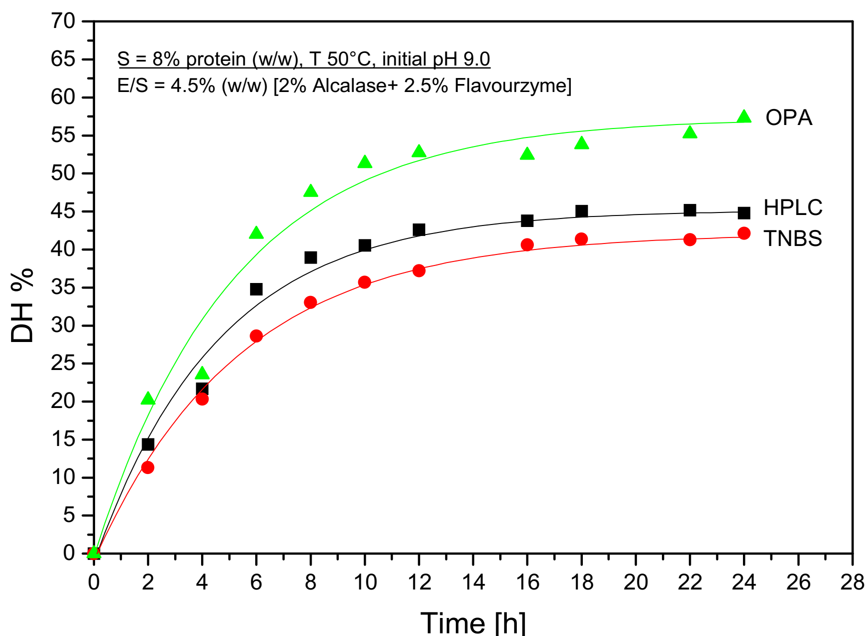



Figure 3.5 Progress curve of hydrolysis of corn gluten meal (CGM) at 8% protein (w/w) with 2% Alcalase and 2.5% Flavourzyme, 50°C and initial pH 9.0. Comparison of determining degree of hydrolysis between TNBS and OPA method with HPLC analysis of their reference.

Table 3.2 The different measurements using TNBS, OPA and HPLC methods of DH% of corn gluten meal (CGM) hydrolysate.

| **Time of hydrolysis [h]** | **DH% of HPLC** | | **DH% of TNBS** | | **DH% of OPA** | |
|---|---|---|---|---|---|---|
| | $\bar{x}$ | **SD** | $\bar{x}$ | **SD** | $\bar{x}$ | **SD** |
| 2 | 14.35 | 0.251 | 16.30 | 0.365 | 17.23 | 0.098 |
| 4 | 21.70 | 0.342 | 22.33 | 0.472 | 23.56 | 0.532 |
| 6 | 34.78 | 0.304 | 38.60 | 0.515 | 40.01 | 0.785 |
| 8 | 38.96 | 0.215 | 39.03 | 0.715 | 40.52 | 0.655 |
| 10 | 40.56 | 0.118 | 39.68 | 0.342 | 42.33 | 0.981 |
| 12 | 42.60 | 0.097 | 41.19 | 0.321 | 43.74 | 0.488 |
| 16 | 43.79 | 0.088 | 41.62 | 0.554 | 44.41 | 0.321 |
| 18 | 45.06 | 0.076 | 42.38 | 0.910 | 46.81 | 0.091 |
| 22 | 45.18 | 0.341 | 41.26 | 0.853 | 47.22 | 0.351 |
| 24 | 44.82 | 0.145 | 42.13 | 0.344 | 47.31 | 0.211 |

$\bar{x}$ = mean value of 10 replicate values

SD = standard deviation

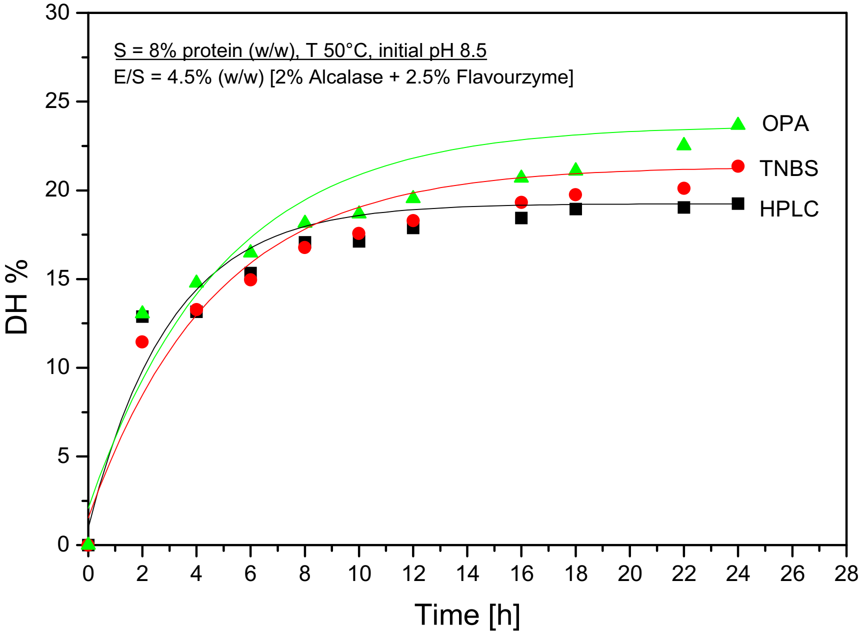



Figure 3.6 Progress curve of hydrolysis of soybean meal (SBM) at 8% protein (w/w) with 2% Alaclase plus 2.5% Flavourzyme, 50°C and initial pH 8.5. Comparison of determining degree of hydrolysis between TNBS and OPA method with HPLC analysis of their reference.

Table 3.3 Illustrates the different measurements e.g. TNBS, OPA and HPLC methods of DH% of soybean meal (SBM) hydrolysate.

| **Time of hydrolysis [h]** | **DH% of HPLC** | | **DH% of TNBS** | | **DH% of OPA** | |
|---|---|---|---|---|---|---|
| | $\bar{x}$ | **SD** | $\bar{x}$ | **SD** | $\bar{x}$ | **SD** |
| 2 | 12.88 | 0.141 | 11.45 | 0.115 | 13.04 | 0.122 |
| 4 | 13.16 | 0.102 | 13.28 | 0.118 | 14.77 | 0.154 |
| 6 | 15.34 | 0.159 | 14.95 | 0.107 | 16.48 | 0.118 |
| 8 | 17.07 | 0.105 | 16.77 | 0.153 | 18.15 | 0.172 |
| 10 | 17.12 | 0.303 | 17.56 | 0.355 | 18.67 | 0.455 |
| 12 | 17.89 | 0.355 | 18.29 | 0.478 | 19.54 | 0.568 |
| 16 | 18.45 | 0.251 | 19.32 | 0.349 | 20.69 | 0.351 |
| 18 | 18.95 | 0.321 | 19.74 | 0.455 | 21.10 | 0.497 |
| 22 | 19.04 | 0.411 | 20.11 | 0.479 | 22.52 | 0.577 |
| 24 | 19.25 | 0.425 | 21.35 | 0.351 | 23.67 | 0.356 |

$\bar{x}$ = mean value of 10 replicate values

SD = standard deviation

The relative standard deviation (RSD) of %DH is defined as SD divided by $\bar{x}$ and multiplied by 100. In Figure 3.7 both of raw materials are shown to compare the accuracy of the two methods. SBM analysis by TNBS method gave a lower RSD than OPA method, with RSD less than 0.5% and 2.9% for TNBS and OPA method, respectively. While for CGM, the RSD of both methods are not different, as around 2.3%.

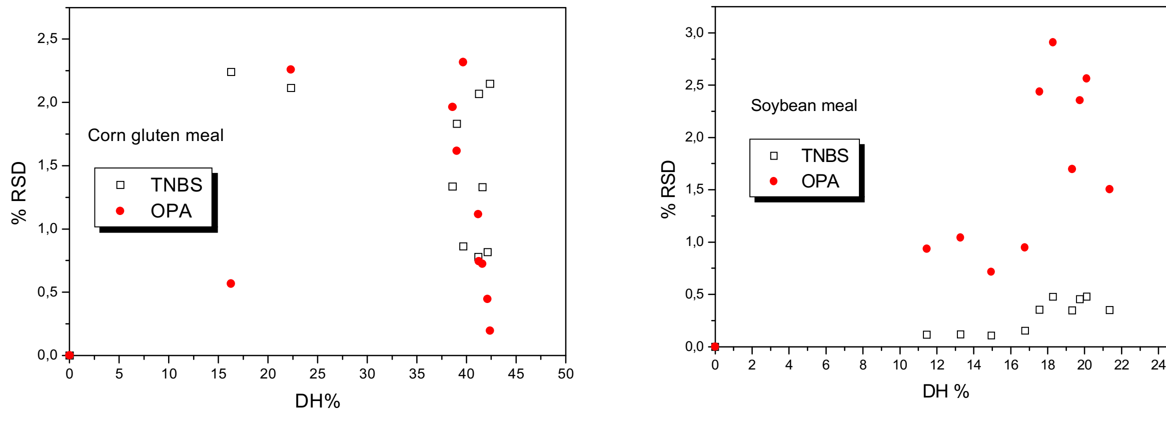



Figure 3.7 Relative standard deviation (RSD) between OPA and TNBS method to determining degree of hydrolysis (DH) for corn gluten meal (CGM) and soybean meal (SBM) hydrolysates.

Figure 3.8 illustrates correlation analysis of TNBS and OPA methods, that depends on raw material. The correlation between OPA and TNBS methods for SBM ($r$ = -0.9) was better than CGM ($r$ = -0.5). The difference in correlation was due to the difference of the amino acid compositions of the protein raw materials. This reason was supported in Table 3.4, that the absorption at 340 nm of colored compounds from reaction with glycine, cysteine and lysine was relatively lower. The different content of these 3 amino acids in SBM and CGM were reflected in the content of free amino acids in the hydrolysates. In addition, glycine and cysteine are bounded in peptides to a larger extent than the other amino acids when proteins are hydrolyzed at high DH (Nielson et al., 2001). Thus, the content of lysine was expected mainly explaining.

Table 3.4 Absorption at 340 nm of OPA method reacted with amino acids and peptides (Nielson et al., 2001).

| Amino Acid | OD/ mmol/ 100 ml | mean % |
|---|---|---|
| Glycine | 5835* | 82 |
| Alanine | 7156 | 101 |
| Leucine | 7310 | 103 |
| Phenylalanine | 7107 | 100 |
| Serine | 7075 | 100 |
| Cysteine | 2311* | 33 |
| Methionine | 7067 | 100 |
| Tryptophan | 6776 | 96 |
| Tyrosine | 7147 | 101 |
| Aspartic acid | 7297 | 103 |
| Asparagine | 7808 | 110 |
| Glutamine | 7646 | 108 |
| Lysine | 5814* | 82 |
| Arginine | 7451 | 105 |
| Histidine | 6732 | 95 |
| N-Glycyl glycine | 6869 | 97 |
| N-Glycyl-glycyl glycine | 6099 | 86 |
| N-Leucyl-methionine | 7240 | 102 |
| N-Lysine-phenylalanine | 7280 | 103 |
| N-Glycyl-leucyl-tyrosine | 6432 | 91 |
| Mean value | 7088 | |
| SD | 419 | |

* Not included in the calculation of mean value

Table 3.5 DH determinations of soybean meal hydrolysate and corn gluten meal hydrolysate measured by OPA and TNBS method.

| **Time of hydrolysis [h]** | **Corn gluten meal (CGM)** | | **Soybean meal (SBM)** | |
|---|---|---|---|---|
| | **TNBS DH, %** | **OPA DH, %** | **TNBS DH, %** | **OPA DH, %** |
| 2 | 16.30 | 17.23 | 11.45 | 13.04 |
| 4 | 22.33 | 23.56 | 13.28 | 14.77 |
| 6 | 38.60 | 40.01 | 14.95 | 16.48 |
| 8 | 39.03 | 40.52 | 16.77 | 18.15 |
| 10 | 39.68 | 42.33 | 17.56 | 18.67 |
| 12 | 41.19 | 43.74 | 18.29 | 19.54 |
| 16 | 41.62 | 44.41 | 19.32 | 20.69 |
| 18 | 42.38 | 46.81 | 19.74 | 21.10 |
| 22 | 41.26 | 47.22 | 20.11 | 22.52 |
| 24 | 42.13 | 47.31 | 21.35 | 23.67 |
| ***r* =** | **-0.50** | | **-0.90** | |

***Note*:** the correlation coefficient $r$; it can assume values between +1 and –1. If $r = 1$ or $r = -1$, a functional relationship exists between x and y. If $r = 0$, no relationship exists between x and y (see Annex 9.5).

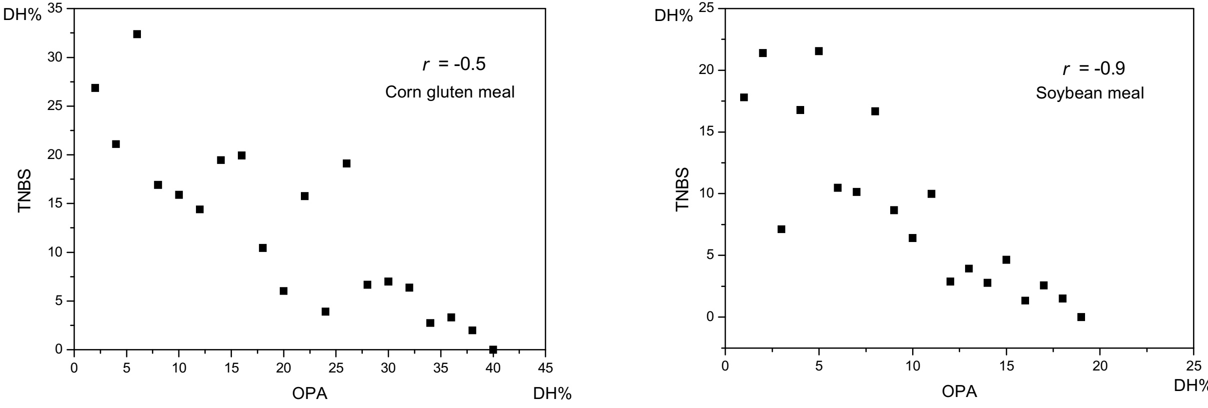



Figure 3.8 Correlation analysis between OPA and TNBS methods to determining degree of hydrolysis (DH) for corn gluten meal (CGM) and soybean meal (SBM) hydrolysates.

The type of raw materials used determines the degree of hydrolysis. OPA method has several advantage over TNBS method. For example, the reagent of OPA forms a stable colored compound with amino groups and was less toxic than TNBS reagent. The TNBS reagent was rather unstable and the solutions need to be kept in the dark to develop a color and was time-consuming. The analysis described in this work was based on the solution TNBS reagent.

Another difference between the two methods were the response time, that the time of OPA reaction was 2 min before measuring absorbance, whereas the TNBS reaction needed an hour. This means the OPA method can be used to monitor DH continuously during hydrolysis.

### 3.4 Summary

Nitrogen analysis was performed the Kjedahl method. The crude protein ($N \times f_N$) content for CGM and SBM were 63% and 50%, respectively. The amount of crude protein was used to calculate the total hydrolysis ($h_{tot}$) of the degree of hydrolysis (DH).

HPLC revered-phase was used for amino acid analysis. Rapid and high sensitivity analysis of only primary amino acids was required. The relative instability of the derivatives made the *o*-phthalaldehyde (OPA) reaction a perfect test for the technique of automated pre-column derivatization.

The different raw materials have different amino acid compositions. The degree of hydrolysis was also analyzed by the OPA and TNBS method. These values were then compared using correlation analysis. The reaction time for the OPA method is 2 min, whereas the TNBS reaction needs an hour. Thus, the OPA method can be used to directly monitor during the hydrolysis reaction.

# 4 Initial Screening of Commercial Proteases

When considering the use of enzymes in hydrolysis processing two key questions arise: 'How pure an enzyme preparation is needed?' and 'How much enzyme must be added?'. Adequate 'purity' and low cost can often be achieved by careful selection of the source organism for enzyme preparation. Thus, fungal and bacterial sources are often used, since these enzymes are cheap to produce and often contain relative few contaminating activities. In many cases, the use of relatively crude extracts is adequate and may be beneficial (Tucker and Woods, 1995).

To quantify or assay the enzyme activity, it is important to monitor the amount of enzyme added during a process. Since enzymes are assayed under optimal conditions, the activity quoted may not represent the true activity of enzyme in actual use. In almost all cases the ratio of enzyme to substrate is enormously greater in these assays than would be adopted in industrial applications of the enzymes and because of this, narrower optima are presented in the plots than would occur in practical applications.

However, the effect of pH, temperature and substrate/product concentrations on enzyme activity were determined. This can sometimes be a response to technical improvements or to economic considerations influencing the choice of substrate components. The screening enzyme activities by endo- and exo-peptidase assays and characteristics of these enzymes by hydrolyzing corn gluten meal (CGM) and soybean meal (SBM) were performed. Information is given concerning their action, reaction optima, sensitivity to inhibitors and activators, and, where available, their stability in practical reaction conditions.

## 4.1 Protease assays

In this section, enzyme activity of various commercial enzymes were determined using protease assay techniques distinguish between endopeptidases and exopeptidases.

In general, enzyme Units (U) or International Unit (IU) is defined as the amount of enzyme that transforms 1 μmole of substrate in 1 minute under a specified set of conditions. The specific activity of an enzyme was the number of enzyme activity units divided by the amount of protein, usually expressed in milligrams. The measurement of specific activity was determined by Bradford assay see Annex 9.2. The specific activity of various commercial enzymes are shown in Table 4.1 (endopeptidases) and Table 4.2 (exopeptidases).

### 4.1.1 The determination of endopeptidase activity

Endopeptidase assays (Annex 9.6) can be used to detect either as many enzymes as possible in crude extracts or to test for the presence of a particular enzyme of well-known specificity. In general, the activity of endopeptidases were assayed using natural substrates, involving the incubation of a protein under a defined set of conditions (Beynon and Bond, 1989).

Table 4.1 Proteolytic activity of endopeptidase

| Enzyme | Type of protease | Enzyme activity [$U^{cas}$/ml] | Specific activity [$U^{cas}$/mg protein] | Unit price[1] [€/kg] |
|---|---|---|---|---|
| **Technical grade** | | | | |
| Alcalase 2,5 L DX | Serine protease | 250 | 6.20 | 15.34 |
| Savinase16,0 L EX | Serine protease | 318 | 7.32 | 15.34 |
| Esperase 8,0 L | Serine protease | 133 | 3.14 | 22.50 |
| **Food grade** | | | | |
| Alcalase 2,4 L | Serine protease | 415 | 4.79 | 19.94 |
| Novo Pro-D | Serine protease | 400 | 11.52 | 25.57 |
| Novozyme FM 2,0L | Serine protease | 290 | 6.48 | 17.90 |
| Neutrase 0,8L | Serine protease | 143 | 4.56 | 15.34 |
| AMI 00.01 | Metallo protease | 85 | 3.08 | 15.34 |
| AMI 00.02 | Cysteine protease | 167 | 3.79 | 15.34 |
| AMI 00.03 | Serine protease | 181 | 4.52 | 15.34 |
| Corolase S50 | Serine protease | 190 | 4.63 | 15.34 |
| Corolase PN-L | Serine protease | 150 | 4.55 | 15.34 |
| Flavourzyme1000L | Aminopeptidase | 75 | 1.32 | 38.35 |
| Extremozyme[2] | Serine protease | 3 | 4.34 | -- |
| Extremozyme[3] | Serine protease | 1 | 3.80 | -- |

[1] Unit price for large volumes

[2] The recombinant carboxy-terminal serine protease from *Thermotoga maritima*

[3] The isolated serine protease from *Thermoanaerobacter keratinophilus sp.nov.*

***Definition of units*** One casein Unit ($U^{cas}$) is the amount of enzyme which digests casein under the above mentioned conditions (Bergmeyer, 1974).

Annex 9.6 provides procedural details for endopeptidase assays, in which casein was used as substrate. This method provided a simple and inexpensive assay suitable for experiments. Thus, crude enzymes were screened using casein for testing general proteolytic activity.

The most commonly used technique was to precipitate the remaining intact substrate and large fragments with trichloroacetic acid (TCA) and removed them by centrifugation. The supernatant were detected directly at 280 nm (Annex 9.6). The results of enzyme activity of various commercial enzymes are shown in Table 4.1. Alcalase, Novo-Pro-D and Novozyme should be considered in the process of enzymatic hydrolysis because they have the highest activity and safety for consumption. The activity of these enzymes were 415, 400 and 290 $U^{cas}$/ml of Alcalase, Novo-Pro-D and Novozyme, respectively.

### 4.1.2 The determination of exopeptidase activity

Natural substrates are rarely employed in the routine assay of exopeptidases, because of the diversity of synthetic substrates and the ease with which an elegant assay, which uses these substrates, can be developed (Beynon and Bond, 1989). The simplest substrates are single amino acids coupled through their α-COOH group to chromogenic amines, such as 4-nitroanilide. Based on the blocking groups employed, substrates can be classified into three categories, namely endopeptidase, aminopeptidase and carboxypeptidase substrates. Endopeptidase substrate will have no free amino- or carboxy-termini, while aminopeptidase substrates will have a free amino-terminus, and carboxypeptidase substrate will have a free carboxy-terminus. Classification of peptidases as endopeptidases or exopeptidases in Chapter 2.3 is based on the results of selection which determine the class of substrate that is hydrolyzed by a given enzyme.

In this work, leucine aminopeptidase EC 3.4.11 LAPU (leucine arylamidase); hydrolyzed L-leucine-p-nitroanilide to from p-nitroaniline (Figure 4.1) which was measured using a photometer at 405 nm (Novo EB-SM-0298.02/01). A representative protocol was given in Annex 9.6. Many nitroanilide substrates will be hydrolyzed spontaneously in basic solutions; thus, preparation of a stock solution in water or in an organic solvent usually facilitates the long-term storage of these substrates. Alternatively, fresh substrate can be prepared daily. Exopeptidases activities are shown in Table 4.2. Flavourzyme exhibited the highest activity of 1000 LAPU/g.

leucine arylamidase

40 °C pH 5

L-leucine-p-nitroanilide + $H_2O$ → β-nitroaniline (Yellow, 405 nm) + L-leucine

Figure 4.1 Kinetic measurement of **l**eucine **a**mino**p**eptidase **u**nit (LAPU).

Table 4.2 Proteolytic activity of exopeptidases

| Enzymes | Type of protease | Enzyme activity [LAPU/g]] | Specific activity [$U^{cas}$/mg protein] | Price[1] [€/kg |
|---|---|---|---|---|
| Flavourzyme 1000L | Aminopeptidase | 1000 | 1.32 | 38.35 |
| Kojizyme 500 MG | Aminopeptidase | 500 | 4.15 | 23.26 |
| Corolase LAP | Aminopeptidase | 350 | 2.68 | 17.13 |

[1] Unit price for large volume

***Definition of units*** One **l**eucine **a**mino**p**eptidase **u**nit (LAPU) is the amount of enzyme which hydrolyzes 1 μmol substrate per minute under the given standard conditions.

## 4.2 Characterization of commercial proteases

The efficiency of the reaction was measured in terms of the enzyme activity which is related to the amount of enzyme. Normally, the defined conditions refer to 25°C which is difficult because the optimum temperature of commercial enzymes (typically around 50 to 100°C) is far removed from the assay temperature of 25°C and the optimum may be temperature dependent.

Otherwise, proteolytic activity in protein hydrolysis cannot be determined by the usual assays because of interference with the substrate. As the activity of enzymes were measured by end-products of the hydrolysis of CGM and SBM, that was mmol amino acids per g protein. These data were calculated after amino analysis by HPLC method.

### 4.2.1 Effect of pH and temperature on enzymatic hydrolysis of corn gluten meal (CGM) and soybean meal (SBM)

The main physical factors affecting activity and stability optima are pH and temperature, although others can have an effect on industrial enzyme reactions. The characteristics of these enzyme present the maximum potential of an enzyme. Hence, it is a useful guide to its application and effectiveness in any given reaction.

It is necessary to optimize the temperature for the hydrolysis reaction. This optimum will represent a compromise between increased reaction rate and minimal enzyme inactivation (Hardwick; 1989). The bell shape curve resulted from increasing rate of reaction at lower temperatures and decline enzyme activity due to denaturation at higher temperatures.

The effect of pH on enzyme catalysis was caused by the ionization of substrate or enzyme, which can affect substrate binding or transformation to product directly or affect enzyme stability (Horton, 1993). However, it is also clear that the ionization of amino acid residues occur outside of the active site itself can also have an effect on the enzyme activity. In most cases, enzymes are active only over a very narrow range of pH values (Fullbrook, 1983).

The temperature tolerance will be the greatest at the optimum pH for many enzymes. However, the effect of pH and temperature must be considered during hydrolysis. Enzymes often exhibit optimum requirements with respect to ionic strength of the reaction mixture. As unlike the case with pH, there was nearly a very broad range of ionic strength over which the enzyme retains appreciable activity. Thus consideration of ionic strength was rarely a key requirement.

Generally, it was advised to select the pH optimum of testing enzyme for each substrate in order to achieve maximum sensitivity. In this work, the commercial proteases of 4.5% E/S (w/w) hydrolyzed corn gluten meal (CGM) and soybean meal (SBM) of 8% protein (w/w) in ionized water at different pH and temperatures. The optimum pH and temperature are listed on Table 4.3. The known optimum pH and temperature of these commercially available enzymes are listed in Annex 9.10. The pH and temperature optimum of CGM and SBM were mostly the same as the reference, except the optimal pH value of Novo Pro-D was around 7-8 of CGM and 7-9 of SBM, while the report of reference is around pH 6.5.

Based on the cost analysis of unit price per ton of substrate and per kg of enzyme converted to products, that Alcalase, Novo Pro-D and Novozyme were considered for endopeptidases and Flavourzyme, Kojizyme and Corolase LAP were considered for exopeptidases. The endopeptidase will be combined with exopeptidase in the overall hydrolysis reaction.

Table 4.3 The optimization of pH and temperature of commercial enzymes hydrolyzing CGM and SBM as substrate.

| Enzyme | Corn gluten meal | | Soybean meal | | Reference[1] | |
|---|---|---|---|---|---|---|
| | pH opt. | Temp. opt. | pH opt. | Temp. opt. | pH opt. | Temp. opt. |
| Alcalase 2,5 L DX | 6.5-9.0 | 45-70°C | 7.0-9.0 | 40-60°C | 6.5-9.0 | 50-70°C |
| Savinase16,0 L EX | 8.0-10.0 | 45-55°C | 8.0-9.0 | 45-50°C | 8.0-10 | 50°C |
| Esperase 8,0 L | 7.5-10.5 | 55-75°C | 8.0-10 | 50-65°C | 7.5-10.5 | 55-60°C |
| Alcalase 2,4 L | 7.0-9.0 | 45-70°C | 7.0-9.0 | 40-60°C | 6.5-9.0 | 50-70°C |
| Novo Pro-D | 7.0-8.0 | 50-60°C | 7.0-9.0 | 50-60°C | ca.6.5 | 55-65°C |
| NovozymeFM 2,0L | 6.5-12.0 | 50-65°C | 7.0-11 | 50-65°C | 6.5-12.0 | 50-65°C |
| Neutrase 0,8L | 5.5-7.5 | 45-55°C | 5.5-6.5 | 40-50°C | 5.5-7.5 | 45-55°C |
| AMI 00.01 | 5.0-8.0 | 55°C | 5.5-8.0 | 50-55°C | 5.0-8.0 | 55°C |
| AMI 00.02 | 5.0-7.0 | 60-70°C | 5.5-8.0 | 55-70°C | 5.0-7.0 | 60-70°C |
| AMI 00.03 | 7.5-9.5 | 60-65°C | 7.0-9.0 | 55-65°C | 7.5-9.5 | 60-65°C |
| Corolase S50 | 7.0-9.0 | 45-70°C | 6.5-9.0 | 50-70°C | 5.0-8.0 | 55-65°C |
| Corolase PN-L | 5.0-7.5 | 40-50°C | 5.5-8.0 | 45-55°C | 5.0-7.0 | 40-50°C |
| Flavourzyme1000L | 5.0-7.5 | 40-60°C | 5.0-7.0 | 45-55°C | 5.0-7.0 | 40-60°C |
| Kojizyme 500 MG | 5.5-7.5 | ~40°C | 5.5-6.5 | 30-40°C | 5.5-6.5 | 30°C |
| Corolase LAP | 6.0-9.0 | 60-70°C | 6.5-9.0 | 55-70°C | 7.0-9.0 | 60-70°C |
| Extremozyme[2] | 9.0 | 70°C | 9.0 | 70°C | 9.0 | 80°C |
| Extremozyme[3] | 10.0 | 70-80°C | 9.0-10 | 70°C | 8.0 | 70°C |

[1] References from product sheet of the companies

[2] From *Thermotoga maritima* (recombinant carboxy-terminal protease)

[3] From *Thermoanaerobacter keratinophilus sp.nov.*

### 4.3 Cost

Cost and suitability restrict the buyer in any attempt to compare enzymes by a simple inspection of the suppliers' enzyme data sheets and specifications. Many enzymes of almost identical price may be described with widely differing numerical unit activities, also confuses the purchaser. It is not safe to compare similar enzymes on their supplier stated activities. It is also not safe to compare enzymes with apparently similar activities products on the basis of their prices. An internally comparable assay, preferably based on the process substrate and conditions, will give a

more reliable comparison. Therefore, when selecting an enzyme, it is helpful to have at least one analytical method established and running in the user laboratory.

The contribution of the enzyme catalyst to an industrial process is generally an extremely small portion of the total input costs (Godfrey and West, 1996). However, it is important that purity and activities were consistent with the cost, and that any side activities have been identified that might influence the processing and product under consideration.

### 4.4 Summary

The effects of pH and temperature on enzyme action depends on the type of substrate. The optimization of pH and temperature from optimal hydrolysis is necessary. On the other hand, the enzyme activity should be determined using the realistic substrate such as CGM or SBM. In conclusion, intelligent screening of proteases do not follow a simple procedure, but requires a technical and economical evaluation.

# 5 Hydrolyses

Prid hydrolysdown is distinguished from hydrolysis and denaturation. Water (in the presence of acid or an enzyme) can be used to break a peptide bond, forming molecules of carboxylic acid and amine (Figure 5.1). The hydrolysis process normally requires high temperatures and is catalyzed by either strong acid or strong base or an enzyme.

Denaturation involves unfolding the protein's secondary, tertiary or quaternary structure (see Chapter 2.3). Denaturation of protein does not affect the primary structure. It does not involve hydrolysis of peptide bonds. Some reagents or factors can cause protein denaturation, these are heat, acids or bases, organic compounds, heavy metal ions, and agitation. When a protein is denatured it looses its biological activity. For example, a denatured enzyme will not longer catalyze its reaction (Barret, 1998).

In this work, all reaction conditions which affect enzyme action were held constant. (Nakai, 1996). Thus, the process of hydrolysis for producing amino acids from raw materials such as corn gluten meal (CGM) and soybean meal (SBM) were determined at the same condition. The enzyme and substrate concentrations were tested to find a critical value in the hydrolysis process. Also, reaction conditions such as pH and temperature were considered in the hydrolysis reaction by using a combination of both enzymes. Enzymes were denatured using high temperature after hydrolysis process to inactivate the enzymes. In addition, heat treatment of the protein prior to hydrolysis will denature the protein structure to make it more accessible to the enzyme attack.

Figure 5.1 Hydrolysis of a dipeptide

## 5.1 Acid hydrolysis

Hydrolysis wiis is most generally used for analysis of total amino acids because it leads to complete hydrolysis with the least destruction. Sulphuric acid is used in some of the earlier investigations on protein structure (Vickery, 1922). At present, the hydrolytic reagent most generally employed is 6 N HCl.

Before any protein or polypeptide can be subjected to amino acid analysis, the sample must be hydrolyzed to cleave the peptide bonds between the amino acids. Acid hydrolysis using hydrochloric acid is the most common hydrolysis method in use as above mentioned, however, the several conditions of this method can partially or totally destroy some amino acids. Whereas, some amino acids were difficult to cleave that required different conditions to achieve complete hydrolysis.

### 5.1.1 Hydrolysis with HCl

#### 5.1.1.1 Reflux method

**Amino acids c**M and SBM (1g) were placed into 250 ml round-bottom flask, then 100 ml 6 N HCl was added, and placed flask in reflux setup. The hydrolysis flask was immersed in an oil bath until the liquid level was just above the oil level. The bath temperature was set at 120 ± 5°C to produce vigorous boiling. Nitrogen was bubbled through the liquid until vigorous boiling was obtained; then nitrogen supply was placed in top of condenser and small flow throughout reflux time was continued (Figure 5.2).

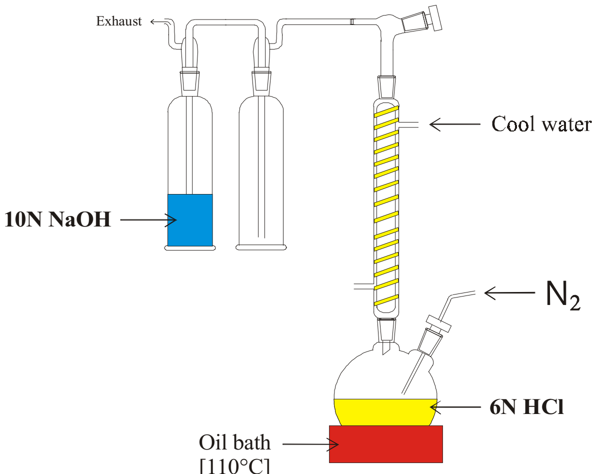



Figure 5.2 Hydrolysis with HCl by reflux method

Samples were hydrolyzed for 24 h to achieve complete hydrolysis. The hydrolysate was filtered, neutralized with 7.5 N NaOH and then washed with distilled water. After which the hydrolysate was evaporated on a rotary evaporator at 50°C. The sample was diluted with methanol (1:5) and frozen prior to amino acids analysis by HPLC (see 3.2).

#### 5.1.1.2 Amino acids composition

Acethod, as mentioned in chapter 3, is the recommended analysis of free amino acids. Figure 5.3 shows that the different native proteins obtained different amino acids. The amount of these amino acids produced by acid hydrolysis of CGM and SBM. The quantity of amino acids composition of CGM and SBM are shown in Table 5.1. CGM and SBM were hydrolyzed 80 and 77% DH, respectively. These data were compared to two references from another laboratories for each raw materials. The references of CGM and SBM were also determined by crude protein by the Kjeldahl method. The quantity of crude protein contents obtained using the reference methods are almost the same.

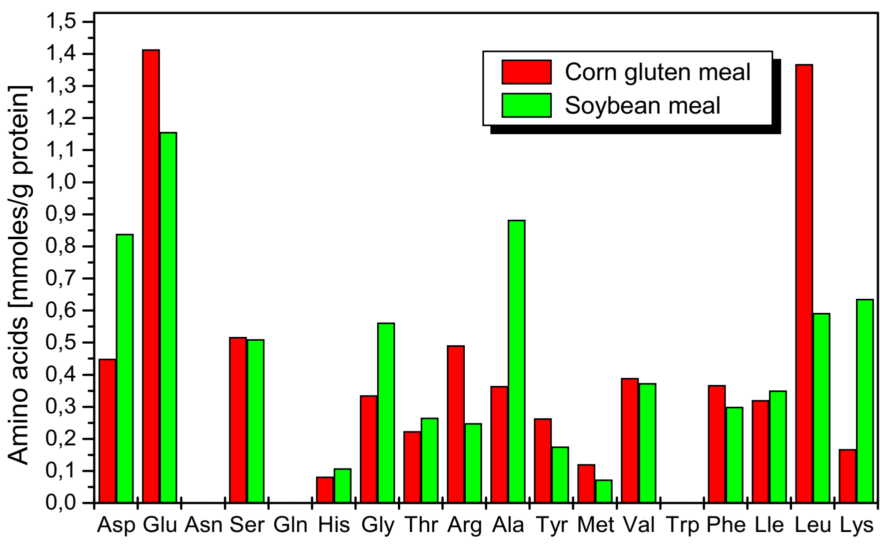



Figure 5.3 Corn gluten meal (CGM) and soybean meal (SBM) obtained different amount of free amino acids using hydrolysis with 6N HCl at 110°C for 24 h.

Table 5.1 compares the amino acids yield of the reference methods and our experiments. Higher yield (around 13% DH of both raw materials) was obtained using the reference methods. This can be attributed mainly to some factors; sources, purity, functional

ingredients, techniques used, duration of hydrolysis to release the amino acids. The hydrolysis time depends on the nature of the linkage in each particular protein (Neuman, 1984). Hence, the hydrolysis time should be optimized in each raw materials. Generally, maximum yields can be obtained after refluxing pure proteins (Noltmann, 1962) and cereals (Tkachuk, 1969) with 6N HCl for 70 h.

Table 5.1 Amino acid composition of corn gluten meal and soybean meal by acid hydrolysis with 6 N HCl at 110°C for 24 h.

| Amino acids [kg/t] | Corn gluten meal | | | Soybean meal | | |
|---|---|---|---|---|---|---|
| | Experiment[1] | References | | Experiment[1] | References | |
| | | I | II | | III | IV |
| 1. Asp | 32.57 | 38.40 | 39.90 | 48.26 | 58.60 | 58.09 |
| 2. Glu | 115.02 | 153.6 | 127.40 | 74.63 | 87.70 | 93.15 |
| 3. Asn | -- | -- | -- | -- | -- | -- |
| 4. Ser | 28.40 | 33.60 | 35.00 | 22.23 | 24.8 | 27.25 |
| 5. Gln | -- | -- | -- | -- | -- | -- |
| 6. His | 7.10 | 11.40 | 15.70 | 7.44 | 13.10 | 12.75 |
| 7. Gly | 12.08 | 16.80 | 18.50 | 16.05 | 21.0 | 21.50 |
| 8. Thr | 14.25 | 21.60 | 22.20 | 13.45 | 19.80 | 19.65 |
| 9. Arg | 48.40 | 16.20 | 20.80 | 19.41 | 23.30 | 36.35 |
| 10. Ala | 16.34 | 19.40 | 19.70 | 31.39 | 21.00 | 24.60 |
| 11. Tyr | 27.08 | 32.40 | 28.40 | 14.40 | 17.30 | 18.75 |
| 12. Met | 10.03 | 16.80 | 17.40 | 4.79 | 6.80 | 5.65 |
| 13. Val | 24.34 | 30.00 | 30.90 | 18.54 | 25.70 | 22.90 |
| 14. Trp | -- | 3.20 | 2.90 | -- | -- | -- |
| 15. Phe | 34.07 | 41.40 | 35.80 | 22.06 | 24.50 | 25.05 |
| 16. Ile | 22.87 | 27.60 | 24.70 | 19.85 | 25.50 | 22.90 |
| 17. Leu | 97.52 | 113.4 | 112.60 | 33.50 | 39.20 | 38.75 |
| 18. Lys | 13.59 | 9.00 | 9.90 | 40.77 | 30.70 | 32.15 |
| **Total** | **503.66** | **565.40** | **561.80** | **386.76** | **439.00** | **460.15** |
| **DH%** | **80** | **94** | **93** | **77** | **88** | **92** |

[1] Raw materials were supplied by Amino GmbH, Braunschweig.

Sources of data:

I CPC International Inc., New Jersey (1974)

II Product data of the Williams Companies, Inc., Illinois (1999).

III The Association of American Feed Control Official Incorporated (AAFCO, 1999)

IV Journal of the AOAC (Vol. 55, No.4, 1972)

Amino acids were released and destroyed at different hydrolysis rates depending on the amino acid composition and characteristics of the sample. Assessment of amino acid composition derived from acid hydrolysis at different time duration (usually 24, 48, and 72 hours) is the most accurate value. Three hydrolysis times were tested to allow selection of specific times for certain amino acids as well as extrapolation to zero time for the most labile amino acids. In most cases, a single 24 hour acid hydrolysis can give adequate information for purposes (Michael et al., 1973). In general, industrial production of amino acids by acid hydrolysis should use citric acid in regulation, according to safety rules for human. The effect of acid hydrolysis on various amino acids was observed in Table 5.2.

Table 5.2 Effects of acid hydrolysis on various amino acids.

| Amino acids | Effects |
|---|---|
| Valine, isoleucine | Bonds are not easily broken |
| Threonine, serine | Slowly destroyed. Serine is a common contaminant |
| Methionine | Partially oxidized during acid hydrolysis |
| Asparagine, glutamine | Converted to aspartic acid and glutamic acid. |
| Tryptophan | Completely destroyed |
| Cystine | Destroyed |

Extensive losses of glutamine, asparagine and tryptophan occured on acid hydrolysis. Glutamine is converted to glutamic acid and asparagine to aspartic acid (Rees, 1946). Tryptophan in crude proteins is destroyed by acid although considerable amounts can be determined if oxygen is rigidly excluded (Noltmann, 1962) and by hydrolysis with hydrochloric acid in quartz vessels in the absence of heavy metals (Monnier, 1950). Tryptophan is not destroyed extensively when heated at 100 to 125°C in 6 to 7 N HCl or $H_2SO_4$ *in vacuo* but degradation occurs in the presence of oxygen, cystine, serine and heavy metals (Canfield, 1963). Other amino acids are destroyed to a lesser extent as demonstrated by decreasing yields with increasing hydrolysis time. Threonine and serine are progressively destroyed (Rees, 1946). There is no general agreement on the rate of destruction (Rees, 1946). Extensive destruction of cystine and cysteine occurs in acid hydrolysis especially when carbohydrate is present (Tkachuk, 1969). Under these conditions, cystine is often determined as cysteic acid in acid in the oxidized protein (Moore, 1963).

### 5.1.2 Summary

The condition of acid hydrolysis was the only standard hydrolysis conditions in amino acid analysis. Several amino acids can be lost severely during conventional acid hydrolysis. CGM and SBM obtained recovery 80 and 77% the yield of amino acids with 6N HCl at 110°C for 24 h. The different raw materials have different amino acids composition.

The most common condition involve treatment with 6N HCl at 110°C for 24 to 72 hours. The optimum time of hydrolysis was determined for each protein as the peptide bonds hydrolyse at different rates. Certain amino acids can be totally destroyed by hydrolysis if not protected. Tryptophan and tyrosine can be protected from chlorine by use of thiol reagents or phenols as scavengers. Cysteine becomes oxidised on hydrolysis, thus, it is necessary to pre-oxidise to cysteic acid before hydrolysis.

Enzyme have been used to avoid hydrolytic losses of amino acids in studies on pure protein (Hill, 1962). However, they cannot replace acids since those amino acids that are released from peptide linkage without destruction can be more accurately determined using acid hydrolysis. In addition, their application to crude proteins is limited (Davies and Thomas, 1973).

## 5.2 Enzymatic hydrolysis using commercially available enzyme

With both supply and long-term use in mind, the enzyme of choice should be supplied in consistent quality and activity. The investment in the plant and process demand reliable and steady performance from the catalysts (Nielsen, 1994). In many situations the choice of enzyme will also be linked to the safety record of industrial use and regulatory approval (in use and in the products to be made) in the countries where the product is made and where it is to be sold.

In applied enzymology, however, not too concerned with the mechanism of the effects, only with the combined observed effects, expressed in a quantitative and practically meaningful way. Data are usually expressed graphically in the form of maximum reaction rate or residual enzyme activity as a function of the variable, in order to interpret the optima values easily.

The screening of enzyme activity was discussed previously in Chapter 4. The interesting enzymes were Alcalase 2,4L, Novo Pro-D and Novozyme of endopeptidases group and Flavourzyme, Kojizyme and Corolase LAP of exopeptidases group. The different enzyme concentrations of these enzymes were varied in the process. In order to understand how an enzyme was likely to react during processing, require an understanding of enzymes kinetics in the different substrates such as CGM and SBM that were determined. Various endo- and exopeptidase were tested in variable combination of enzyme.

### 5.2.1 Protein hydrolysis relative to enzyme kinetics

Kinetics provide a systematic approach for the analysis and quantification of the effect of these parameters on enzyme activity. However, in many cases the mathematical models must assume ideal conditions of the enzyme reaction (Constantinides, 1980). Often these conditions are related to those encountered in practice, and in most cases the kinetic models are a good indication of enzyme action *in situ* (Turker and Woods, 1995).

Kinetic considerations usually are applied in two cases, either for a qualitative description of the curve shapes and the composition of the resulting amino acids or for a quantitative assessment of the effect of changing the hydrolysis conditions such as temperature or enzyme concentration. The quantitative relationship between (the degree of hydrolysis) DH and time, which the hydrolysis curves represent in graphical form cannot be predicted theoretically but must be determined by experiment (Adler-Nissen, 1993). The shape of reaction curve often followed the integrated Michaelis-Menten equation.

The kinetic basis for this effect is that the peptides resulting from the initial attack of the proteases on the protein molecules also act as substrate for further degradation to smaller peptides and amino acids. This process results in substrate competition between the original protein, S, and the peptides, P, as shown in the simplified scheme below.

$$S \xrightarrow{\upsilon_1} P \xrightarrow{\upsilon_2} R$$

This situation will result in a rapid build-up of P, the intermediary product. The substrate saturation with respect to P develops. This model complies qualitatively with endoprotease-protein systems investigated to date (Nagodawithana and Reed, 1993). For example, the model explains why unconverted protein is still generally observed, even after quite extensive hydrolysis.

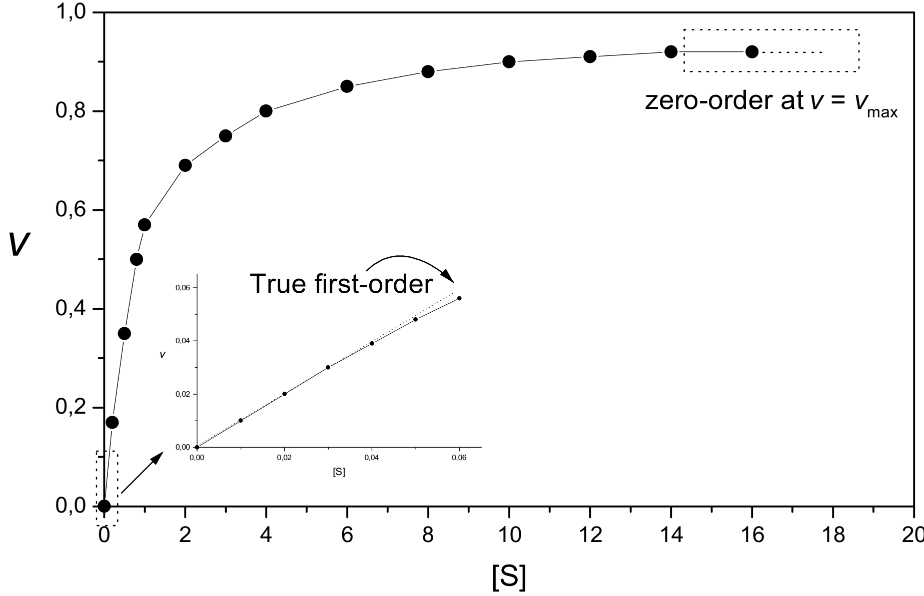



Figure 5.4 $v$ versus [S] plot over a wide range of [S].

Three district regions where the velocity responds in a characteristic way to increasing [S] (Figure 5.4). At very low substrate concentrations (e.g. [S]<0.01 $K_m$), the $v$ versus [S] curve is essentially linear; that is the velocity (for all practical purposes) is directly proportional to the substrate concentration. This is the region of *first-order kinetics*. At very high substrate concentrations (e.g. [S]>100 $K_m$), the velocity is essentially independent of the substrate concentration. This is the region of *zero-order kinetics*. At intermediate substrate concentrations, the relationship between $v$ and [S] follows neither first-order nor zero-order kinetics. The characteristics of the first-order and zero-order regions are described below.

#### 5.2.1.1 Zero- and first-order kinetics

Corn gluten mchanism model that leads to the Michaelis-Menten equation of enzyme kinetics:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

***Reaction at high substrate concentration***:

The maximum reaction velocity, which is attained only at high substrate concentration. Usually in an enzyme reaction, [S] > $E_T$, so that [ES] is formed completely, which means that [E] is virtually zero, so that E = [ES].

$$v_0 \equiv \left(\frac{d[P]}{dt}\right)_0 = k_2[ES] \cong \frac{k_2[E]_T[S]}{K_m + [S]} \quad \text{(steady - state \& initial rate)}$$

In the above equation, $[E]_T$ is the total enzyme concentration that is initially delivered to the system. $K_m$ is termed the Michaelis constant and is defined as

$$K_m \equiv \text{Michaelis constant} \equiv \frac{k_{-1} + k_2}{k_1}$$

As the [S] becomes very large, the velocity of the reaction will not increase indefinitely, but, for a fixed amount of $[E]_T$ will reach a limiting value termed $V_{max}$ , the maximal velocity. That at large [S], for [S] >> $K_m$, $V_{max}$ is reached at saturation.

$$v_0 = V_{max} = k_2[E]_T = \text{constant} \rightarrow v_0 = \frac{V_{max}[S]}{K_m + [S]}$$

<u>Note</u>: when [S] >> $K_m$, $v_0$ approximately equals $V_{max}$, we have <u>zero-order</u> kinetics, the rate of reaction does not depend on [S], but rather on the rate of "catalytic conversion" and release of the product by the enzyme-substrate complex (ES $\rightarrow$ E + P).

***Reaction at low substrate concentration***:

At low substrate concentration this curve is virtually linear, so that the initial reaction velocity is directly proportional to substrate concentration. This means that the reaction is now of the first order type. It shows that under controlled conditions, enzymes can be used to measure directly the concentration of their substrates. This kinetic is important when evaluating the efficiencies of enzymes activities.

The [S] concentration at which the initial rate reaction reaches half (1/2) of the maximum is defined as:

$$[S] \equiv K_m \quad \text{when} \quad v_0 = \frac{V_{max}}{2}$$

Since $K_m$ is directly proportional to $k_2$, this means that an enzyme with a small $K_m$ value will achieve maximal catalytic activity at low [S]. Additional interesting information is revealed if the limiting case of small [S], i.e. $[S] << K_m$. If the substrate concentration is so small such that $[S] << K_m$, then $K_m + S$ in the denominator of the Michaelis-Menten [MM] equation approximately equals $K_m$. If the denominator of the MM equation merely by $K_m$, the MM equation becomes

$$v_0 \approx \frac{V_{max}[S]}{K_m} = \left(\frac{k_2[E]_T}{K_m}\right)[S] \Rightarrow \text{First - order kinetics for } [S]$$

Refer to the Michaelis-Menten plots of $v_0$ versus [S] that rate of reaction is first-order (linear) at small [S] and zero-order at large [S] (steady state).

These kinetic studies come from various references, for example, Adler-Nissen, 1986; Nagodawithana and Reed, 1993; Copeland, 2000; Turker and Woods, 1995; and Segel, I. H., 1975).

### 5.2.1.2 One-by-one versus zipper reaction

Many protein are more easily hydrolyzed if they are denatured by e.g. heat treatment. This effect of denaturation was considered in the heat treatment prior to hydrolysis process. The peptide bonds of substrate are available attacked by protease, which explains the increased rate at which denatured proteins are hydrolyzed.

The relatively high resistance to proteolysis of many globular proteins led to propose a scheme by which an initial reversible denaturation step is essential for hydrolysis to occur (Linderstr∅m-Lang, 1952).

$$\text{Native} \underset{v_{+0}}{\overset{v_{-0}}{\rightleftharpoons}} \text{Denatured} \xrightarrow[v_{I}]{\text{enzyme}} \text{Intermidiate} \xrightarrow[v_{II}]{\text{enzyme}} \text{End product}$$

The reaction rate of denaturation is: $v_0 = v_{+0} - v_{-0}$

This type of reaction is call one-by-one by Linderstr∅m-Lang, indicating that a particular protease molecule degraded one substrate molecule at a time. In the opposite case, where $v_1 << v_0$, the native protein molecules are rapidly converted to the intermediary form which is then more slowly degraded to end products (‘zipper reaction’). Real proteolytic reactions are

of course intermediates between these two types. The effect of pre-denaturation on the hydrolysis reaction is also derived from the above scheme.

Conventional reaction kinetics for sequential reactions predicts that the reaction mixture contains both unconverted substrate and end products, but negligible amounts of intermediate products. It should also be mentioned that the composition of the soluble fraction of the one-by-one type depends on the proportion between native and denatured protein in the initial material. If the initial attack is of a much higher rate than the subsequent degradation to peptides, it will appear as if all the native protein is denatured and unfolded in the very beginning of the hydrolysis

The one-by-one versus zipper reaction models were originally derived for the hydrolysis of native globular proteins (Adler-Nissen, 1986). These predictions are summarized in Figure 5.5. If the hydrolysis were not of the one-by-one type, large peptides would also be present, and consequently the yields and fluxes obtained would be lower.

| **Degree of hydrolysis (DH)** | | **Low DH** | **High DH** |
|---|---|---|---|
| one by one type | Soluble | | |
| | Insoluble | | |
| Zipper type or denatured protein | Soluble | | |
| | Insoluble | | |

Figure 5.5 Composition of protein hydrolysates as a function of the type of initial degradation. Circles denote native protein, large rectangles denote denatured protein, small rectangles denote peptides and smaller denote amino acids, black triangles denote the hydrolysis residue which remains insoluble even at high DH values (Adler-Nissen, 1976).

#### 5.2.1.3 Time course of the hydrolysis

After mixing an enzyme with its substrate in solution, the amount of product over time was measured. The time course of product appearance and substrate depletion were well modeled by a linear function up to the time (O'Meara; 1985). By varying solution conditions, it is possible to alter the length of time over which an enzyme-catalyzed reaction will display

linear kinetics. It is assumed that the reactions velocity was measured during this early phase of the reaction, which means that from here $v = v_0$, the initial velocity.

Throughout the progress curve, the velocity was changing as the substrate concentration available to the enzyme continues to diminish. The data points from a plot of $y$ versus $x$, hence, the instantaneous velocity ($v = d[P]/dt = -d[S]/dt$) could determined of the enzyme progress curve (Cornish-Bowden, 1994). This plot can be applied to the Michaelis-Menten equation.

#### 5.2.1.4 Practical applied kinetics

When considering the industrial uses of enzymes or even the purchase of a few milligrams of a purified enzyme for academic research, the same type of questions arise. How much enzyme is needed? How long is the reaction time? What are the concentrations of substrates required? What physical conditions of temperature, pH, ionic strength should be used for optimal reaction? How much is it going to cost? Enzyme kinetic can provide some useful answers to all these questions.

Essentially, when considering the use of enzymes on an industrial scale, it is attempting to justify their utility or viability against a background of hard economic reality. The cost effectiveness of an enzyme-catalyzed process is derived directly from the relationship between the additional cost of the enzyme system involved and either the final added value in the product obtained (Godfrey and West, 1996). These result to overall cheaper processing and higher product yield.

### 5.2.2 The operation of enzymatic hydrolysis

The protein hydrolysis were carried out using in 0.1 L reactor and reproduced in 4 L batch reactor. These scales were used for the laboratory experiments. The process of hydrolyses were carried out as batch reactions.

In principle, the hydrolysis reaction in a well-stirred reactor is scale independent (Adler-Nissen, 1986). The reaction proceeds with negligible change in the density. pH was not adjusted during the reaction. The reactions were carried out iso-thermally, and rapid heating was needed only occasionally at the end of the reaction to heat inactivate the enzyme. For this reason, the batch hydrolysis reaction can easily be scaled up to industrial size. The major

differences between laboratory and pilot plant or industrial scales lie in the separation, recovery and refining of amino acids, after the hydrolysis reaction has been terminated.

#### 5.2.2.1 Preparation of the reaction mixture

For the used specified substrate and enzyme, the following test parameters must be defined:

- the mass of hydrolysis mixture (M, in g)
- substrate concentration, S in % (w/w) of protein ($N \times f_N$)
- enzyme-substrate ratio, E/S. The use of weight percent of protein as substrate is common and quite acceptable if the activity of the enzyme preparation is indicated.
- pH
- temperature, T in °C

From the specified value of M and the hydrolysis parameters, the hydrolysis mixture can be prepared. The substrate can be weighed and mixed with the water directly in the hydrolysis vessel. In laboratory, the amount of enzyme used was small, and it was therefore necessary to dilute the enzyme preparation prior to use, in order to ensure that it can be transferred quantitatively to the hydrolysis mixture. On pilot plant and industrial scale, dilution was not necessary and should be avoided (Novo Nordisk, 1978b).

The protein content of the raw material was calculated using the formula below (all masses are either in g or kg):

**M** : the mass of hydrolysis mixture.

**MP** : the mass of protein in the hydrolysis experiment:

$MP = M \times (S\%/100)$

**$M_{enz}$** : the mass of the enzyme solution.

After homogenization ($M - M_{enz.}$) g was weighed and placed into the hydrolysis vessel. The temperature was raised to 50 °C, and pH to 9.0 was adjusted in the hydrolysis mixture by adding initial volume of base to raise pH in the substrate to the pH value of the hydrolysis reaction. The solution mixture was agitated at 400 rpm. When the hydrolysis mixture was in equilibrium around 45 min, the enzyme solution was added quickly. The enzyme solution was prepared freshly by dissolving **ME** (the mass of enzyme) g enzyme in deionized water to a total mass of $M_{enz}$ g. As the enzyme-substrate ratio (E/S) give as activity units per g of substrate and weight percent that calculates: $ME = \frac{E/S\%}{100} \times MP$

#### 5.2.2.2 Procedure of the hydrolysis

In enzymatic hydrolysis, endopeptidases were always used, but occasionally the endopeptidases were combined with exopeptidases in order to achieve a more thorough degradation.

***Enzymes*** The following food-grade proteases were available for the hydrolysis of vegetable proteins:

| | | |
|---|---|---|
| **Endoproteases:** | Alcalase 2,4 L | (Novo Nordisk, Denmark) |
| | Novo Pro-D | (Novo Nordisk, Denmark) |
| | Novozyme FM | (Novo Nordisk, Denmark) |
| **Exopeptidase/** | | |
| **endoprotease complex:** | Flavourzyme 1000L | (Novo Nordisk, Denmark) |
| | Corolase LAP | (Röhm Enzyme, Damstadt) |
| | Kojizyme 500MG | (Novo Nordisk, Denmark) |

***Hydrolysis*** The initial pH of the substrate mixture was different between corn gluten meal and soybean meal. CGM will typically have a slightly acid pH ~4 and SBM typically have a pH around neutral when suspended in water. Thus, with SBM less salt was added to the protein hyrolysate and only two pH adjustment was needed instead of four. The initial hydrolysis with endoprotease was used before hydrolysis with exopeptidase:

1. First hydrolysis with endoprotease starting at pH 9.0

| | | |
|---|---|---|
| Substrate conc. | : | 2-12 % (w/w) protein in aqueous suspension |
| Enzyme conc. | : | 0.5-2.0 % of substrate protein |
| Temperature | : | 50°C |
| Time | : | 15-30 minutes |

The pH should be allowed to drop by itself during hydrolysis, typically to around 7.

2. Second hydrolysis with exopeptidase pH will typically drop further to around 6-5.

| | | |
|---|---|---|
| Temperature | : | 50°C |
| Enzyme conc. | : | 1.0-2.5 % of substrate protein |
| Time | : | 8-24 hours |

If a higher degree of hydrolysis (DH) was desired, the pH will be lowered to 5.0 after a few hours of hydrolysis. The solution should be readjusted pH to around 7 for a few minute. The long reaction times requires that the reaction mixture is preserved or stabilized to prevent microbial spoilage.

The reaction mixture should be stirred adequately to facilitate hydrolysis. Suction of air into the reaction mixture, which could lead to excessive formation of foam, should be avoided.

***Sampling*** Samples of 2-10 ml were taken every 2 h of the 24 h hydrolysis. The enzyme was inactivated by placing the sample in a water bath at 90°C for 15 min. The sample was centrifuged, and supernatants were diluted 1:5 with ethanol before supernatant was analyzed for amino acids by HPLC method or frozen for later hydrolysis.

The progress of hydrolysis can be followed by various analytical methods. As the degree of hydrolysis (DH) was measured mainly by HPLC analysis of amino acids. The determination of the number of free amino groups such as TNBS or OPA methods can be analyzed alternatively.

***Inactivation of enzymes*** The enzymes were inactivated at 85°C for 5-10 minutes after hydrolysis has been completed. It should be noted that protein substrate rich in starch and other carbohydrates will be broken down to reducing sugars during hydrolysis with Flavourzyme. CGM will often be browned during prolonged heat treatment due to the formation of Maillard products (Novo Nordisk, B163k-GB).

***Further processing*** After the enzyme process and any inactivation stage is needed. A separation stage was needed in order to remove unhydrolyzed or undissolved material prior to purification of amino acids intend.

***Comments*** The batch process was better than the continuous process in protein hydrolysis because:

1. the problem of maintaining hygienic conditions in the CSTR is sometime insurmountable in practice.
2. the batch reactor is much easier to control and its kinetic can easily be investigated in laboratory experiments.
3. the use of a crude enzyme containing both enzymes was more practical.

***In summary*** A laboratory hydrolysis experiment is adequately specified if the following test parameters are known:

- the substrate and enzyme
- the hydrolysis parameters (S, E/S, pH and T)
- the mass of hydrolysis mixture (M)
- degree of hydrolysis (DH)
- inactivation conditions (pH, T and time, t)

### 5.2.3 Enzymatic hydrolysis of corn gluten meal (CGM) and soybean meal (SBM)

The nutritional value of amino acids were normally maintained or increased by enzymatic hydrolysis. This was carried out under mild reaction conditions to defined peptides and amino acids fractions for a pharmaceutical or cosmetic usage.

In this work, a combination of enzymes between endo-and exopeptidase was studied. Alcalase 2,4 L (endopeptidase) was combined with Flavourzyme 1000L (exopeptidase). Mode of action, endopeptidase works by cleaving peptide bond in the interior of polypeptide chains, whereas exopeptidase cleave off amino acids from the end of polypeptide chains (see Chapter 2.3.2). The enzymes were diluted by 1 to 10 to reduce its viscosity for ease of addition to the reactor.

Corn gluten meal (CGM) and soybean meal (SBM) were supplied by Amino GmbH, Braunschweig. These substrate were varied 2-12% by weight protein slurry and were diluted with deionized water (Adler-Nissen, 1977). The combination of both enzymes (Alcalase and Flavourzyme), was 1 to 1 and enzyme concentrations were varied from 3-9% based on weight percent protein substrate. The degree of hydrolysis (DH), defined as the percentage of peptide bonds cleaved (see Annex 9.8) was calculated after HPLC analysis of amino acids.

#### 5.2.3.1 Substrate concentration

Substrate concentration [S] is given as weight percent protein ($N \times f_N$) of the total mass of the reaction mixture at the beginning of the reaction (see 5.2.2.1). A fixed enzyme concentrations was used, as the reaction proceeded. The available substrate becomes a contributory limiting factor in the reaction rate. It was important to look at the effect of substrate concentration on the initial rate of the reaction. In this case, the initial velocity measurements are valid only in the range of substrate depletion between 0 and 10% of the total initial substrate concentrations (Copeland, 2000).

Figure 5.6 illustrates the time course of the combination of both enzymes-catalyzed reaction observed at different starting concentrations of substrate; the velocities for each experiment were measured as the slopes of the plots of amino acid concentrations [P] versus time. In Figure 5.7 replots of these data are give for the initial velocity ($v$) as a function of [S], the starting concentration of substrate. Thus, the velocity was apparently saturated at high substrate concentrations of CGM, S = 8%, at the velocity after 8 h hydrolysis was 0.0504 mmoles amino acid per minute.

Below 4-6%, however, the substrate concentration was presumably too low to ensure substrate saturation. The kinetics were determined by substrate saturation throughout the reaction. This suggests that a zero order kinetic scheme should be applied rather than first order kinetics (see 5.1.2.2). However, the kinetic model was applied, which was not supplied by measurements of $K_m$ and therefore remains speculative (Adler-Nissen, 1978b).

The amino acid concentrations and the rate of hydrolysis increased with increasing substrate concentrations. This arise due to the mechanism of enzyme action in which the enzyme forms a one-to-one stoichiometric complex with its substrate [ES] and it was only this complex that can breakdown to give the product [P] (Tucker and Woods, 1995). Thus, it was expected that for such reactions the initial velocity will be directly proportional to [S] only at the lower range of [S] and when [S] increases the initial velocity approaches a maximum ($V_{max}$). It was usual to determine initial velocities in kinetic studies since, velocity was not always linear with time (Copeland, 2000).

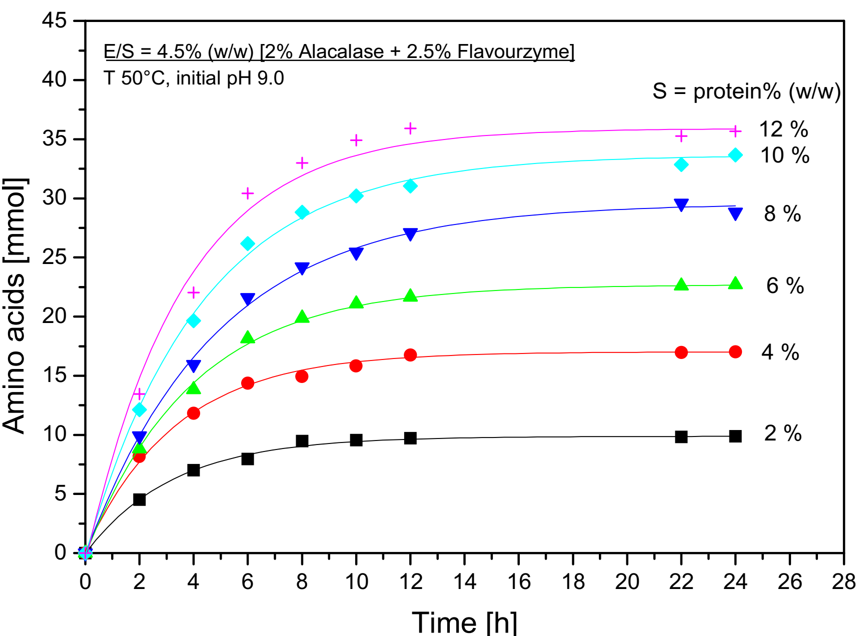



Figure 5.6 Progress curve of hydrolysis for corn gluten meal (CGM) with different starting substrate concentrations by a constant enzyme concentration was the combination of both enzymes (2% Alcalase and 2.5% Flavourzyme).

In addition, CGM was a low soluble substrate, the relation between the initial reaction rate and the substrate concentration was linear because the enzyme concentration was low (E << S). The substrate surface was covered partly by adsorbed enzyme (McLaren and Packer,

1970). In this case, the reaction rate will level off at high substrate concentrations. As the substrate concentration becomes very high, the velocity of the reaction will not increase indefinitely, but, for a fixed amount of enzyme will reach a limiting termed $V_{max}$.

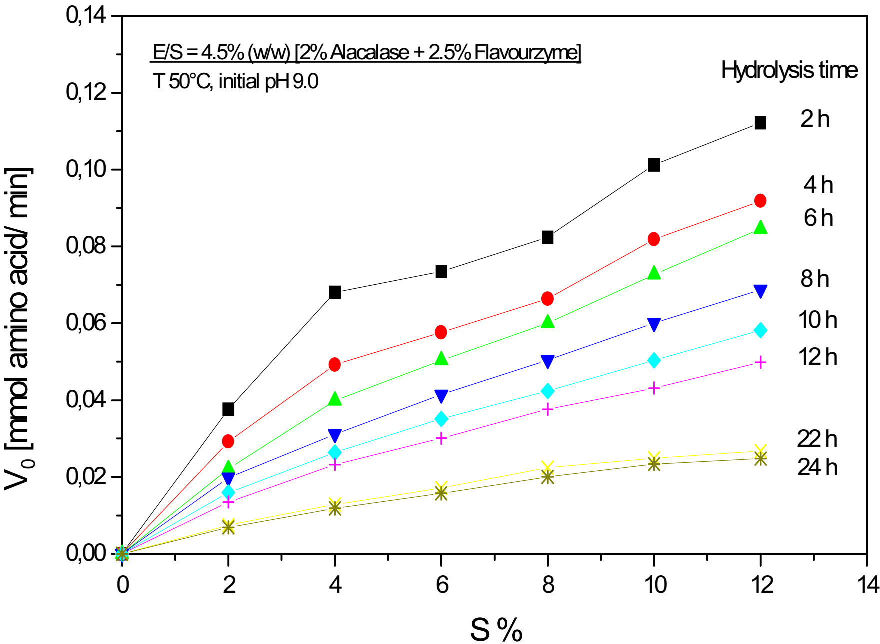



Figure 5.7 Michealis-Menten plots for corn gluten meal (CGM) with different starting concentrations of substrate (these data were calculated from Figure 5.6).

In practice, the range of substrate concentrations was limited by viscosity. Hydrolysis of soybean meal (SBM) was carried out at S ≥ 10% rarely gave reproducible hydrolysis curves (Figure 5.8). The velocity of hydrolysis of SBM, S = 10% obtained 0.0026 mmoles amino acid per minute after 8 h hydrolysis. Although, SBM was more soluble and lower viscosity than CGM. The rate of hydrolysis for CGM was higher than SBM according to be about 19 folds higher at the substrate saturation of each raw material.

In case, the dilution mixture was affected the hydrolysis rate. The hydrolysis rate was increased more at lower substrate levels. The highest degree of hydrolysis (DH) obtained 60% at S = 2% (w/w), as shown in Figure 5.9. Whereas at S = 12% (w/w) was obtained only 35% DH. Generally, the reaction will go to the limit of the degradative capacity of the enzyme (Godfrey and West, 1996). There was also a little effect of product inhibition on reaction rate. However, the economic penalties of large volume reactions and subsequent concentration should be considered carefully when choosing the substrate concentration.

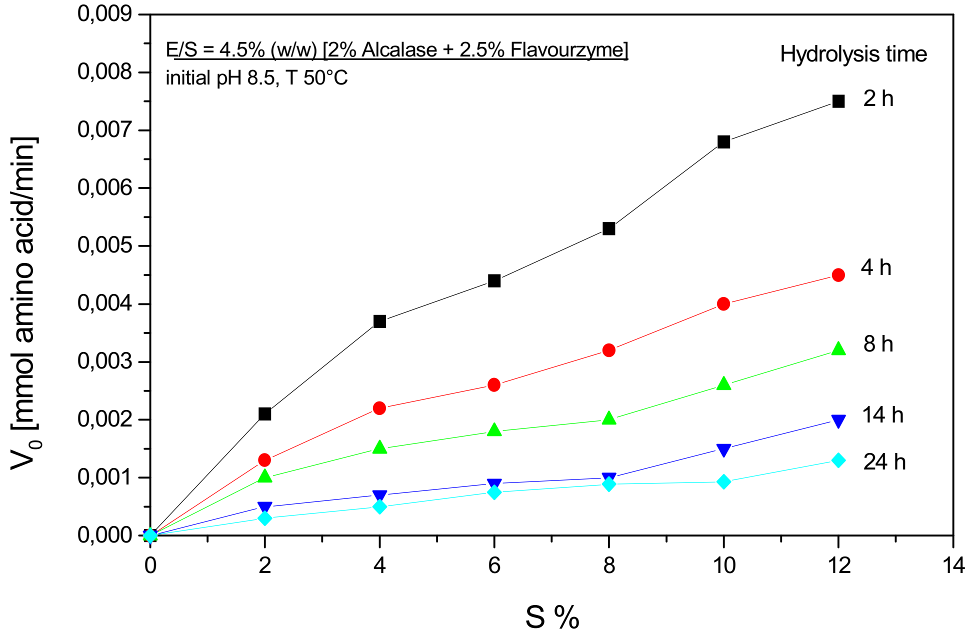



Figure 5.8 Michealis-Menten plots for soybean meal (SBM) with different starting concentrations of substrate.

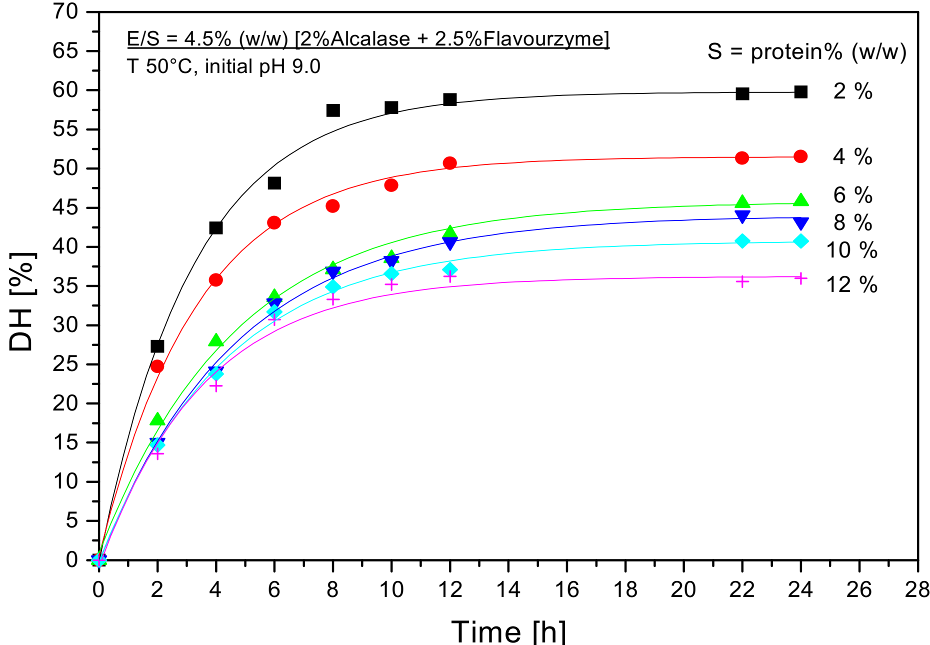



Figure 5.9 Progress curve of hydrolysis for corn gluten meal (CGM) with different starting substrate concentrations. Enzyme concentration is the combination of both enzymes (2% Alcalase and 2.5% Flavourzyme) (data are related from Figure 5.6).

#### 5.2.3.2 Enzyme concentration

The enzyme-substrate ratio, i.e. the concentration of enzyme relative to the concentration of substrate, is usually more characteristic of the reaction velocity than the enzyme concentration per se. This is because protein hydrolysis processes are often carried out at relatively high substrate concentrations where substrate saturation is prevailing (Adler-Nissen, 1982). Thereby, the conversion rate expressed the increase amino acids per unit of time, which is proportional to the mass ratio of enzyme to substrate. In addition, this ratio also forms the basis for assessing the contribution to the variable cost of using the enzyme. Consequently, the enzyme-substrate ratio, abbreviated E/S, was preferred to express the amount of enzyme used in a protein hydrolysis process. E/S can most simply be given as weight percent.

This experiment, corn gluten meal (CGM) was used at the standard value of 8% protein (w/w). CGM was hydrolyzed using the combination of both enzymes, Alcalase to Flavourzyme (1:1) with initial pH 9 and 50°C (see 5.2.2.2). After 24 h hydrolysis, the following degree of hydrolysis (DH) values were reached 33%, 37.8%, 45.2%, 46.3% and 49.2% respectively for varying enzyme concentration values of 3, 3.7, 5, 7 and 9 % based on weight of protein substrate, while the progress DH was negligible increase (Figure 5.10). Figure 5.11 shows the rate of reaction as a function of E/S%. The rate of hydrolysis increased linearly with increasing enzyme concentrations but was limited to 5% E/S. If enzyme concentration was more 5% E/S, the progress curve of reaction velocity versus % E/S will be downward to be constant as enzyme increase. This was indeed the case for any value of time. This result suggests that enzyme-substrate ratio in a process should be limited to lower than 5%. 3% E/S was the lowest enzyme concentration that was recommended in the hydrolysis process.

In general, it should work at enzyme concentrations that are very much lower than the substrate concentration. This was true for most cases provided that [E]<<[S] (i.e. substrate levels exceed enzyme levels), there are often exceptions that must be considered. A plot of reaction velocity (*v*) against enzyme concentration can give a curve showing progressive inhibition (downward) as enzyme increases. A downward curve as depicted in Figure 5.11 was the more common occurrence, showing saturation at higher rates. Alternatively, insufficient levels of an essential co-factor or a dissociable inhibitor would give similar effects.

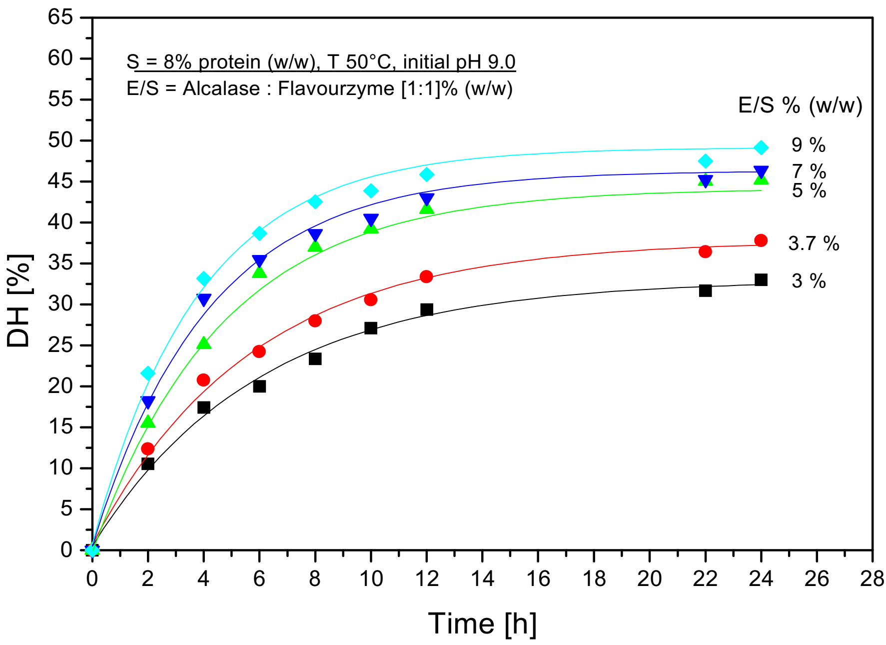



Figure 5.10 Progress curve of hydrolysis of corn gluten meal (CGM) with different enzyme-substrate ratio using the combination of both enzymes (Alcalase to Flavourzyme, 1:1).

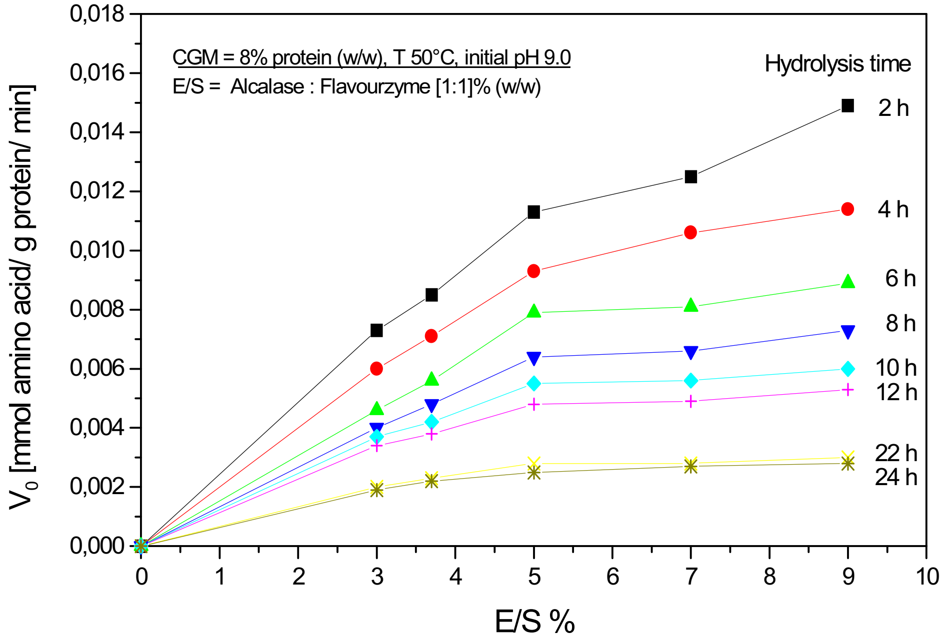



Figure 5.11 Michaelis-Menten plots for corn gluten meal (CGM) with different enzyme-substrate ratio using the combination of both enzymes (Alcalase to Flavourzyme, 1:1).

The reasons for reduced activity with time are often complex but a major cause, presuming a closed system, was obviously depletion of substrate (Horton, 1993). During the early stages of the reaction when [S] was constant, the enzyme reaction should be zero order with respect to product [P] formation (see 5.2.1.2). Other reasons for the reduced enzyme rate with time were inhibition by end-product.

These data show the proportionality between velocity and enzyme concentrations in accordance to the Michealis-Menten equation. In case of the specificity of proteases acting on protein varied with E/S in a manner that the enzyme can hydrolyze a few peptide bond at low E/S. This behavior was denoted by the hypothesis of narrowing specificity. Conversely, when E/S was increased the rate of splitting certain peptide bonds appeared to increase proportionally to E/S (Adler-Nissen, 1986).

Like with CGM, the effect of enzyme concentration on reaction rate of soybean meal (SBM), resulted to a rate of hydrolysis which also was linearly at E/S lower than 5%. The reaction rate decreased during the later period of the hydrolysis (Figure 5.12). However, the velocity of CGM was higher than SBM around 3 folds when 5% E/S was used after 8 h hydrolysis. The reason for lower productivity in SBM was the inactivation of the enzyme, due to activator content of raw materials.

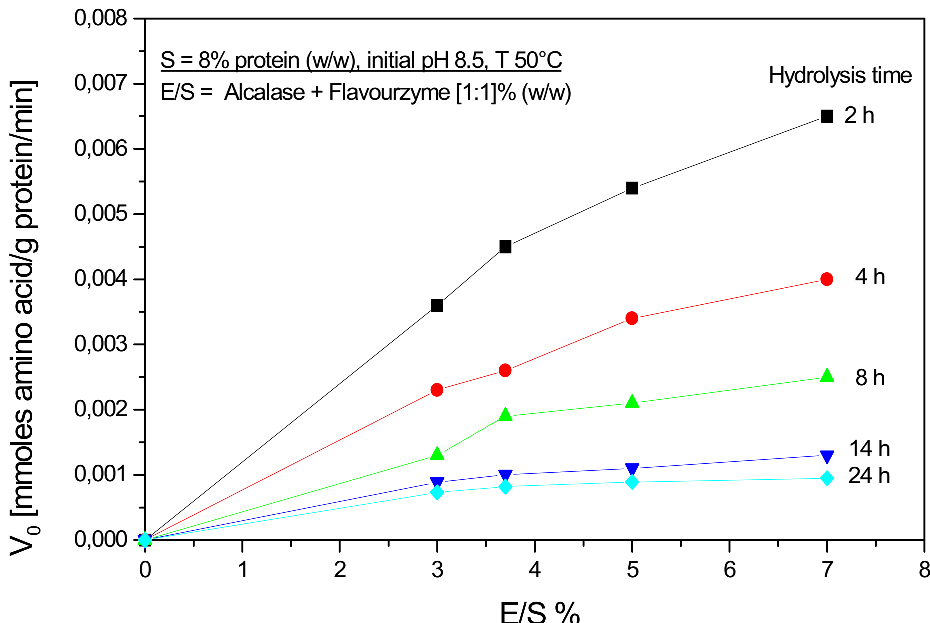



Figure 5.12 Michaelis-Menten plots for soybean meal (SBM) with different enzyme-substrate ratio using the combination of both enzymes (Alcalase to Flavourzyme, 1:1).

#### 5.2.3.3 Temperature effects

In hydrolysis processes, the optimum temperature for a reaction often should be identified in the context of the extent of substrate transformation to product (Adler-Nissen, 1986). The effect of temperature on the reaction rate and overall product yield of an enzyme reaction can possibly be due to several factors, some of which are summarized in Table 5.3. The reactions will certainly be modified by the temperature stability of the enzyme, and this might be the dominant influence. Therefore, the temperature stability of an enzyme was considered resistance to heat inactivation (Godfrey and West, 1996).

Experimentally, corn gluten meal, S = 8% protein (w/w) was hydrolyzed by the combination of both enzymes, the first hydrolysis with 2% Alcalase (w/w) at initial pH 9 and 50°C. After 30 minutes of reaction, the pH should be allowed to drop to around 7. Flavourzyme was added of 2.5% E/S (w/w) to the reaction mixture, and temperatures was varied at this second hydrolysis.

Figure 5.13 shows a series of progress curves of hydrolysis on various temperatures and illustrates enzyme stability for a typical commercial enzyme reaction. After 24 h hydrolysis, the samples were analyzed for amino acids composition by HPLC method. The progress curve shows the two different effects of temperature, namely an increase in initial velocity over the period 0-2 h and inactivation of the enzyme due to its temperature-labile protein nature. When the curve was parallel to the time axis it can be assumed that the enzyme has lost its activity (Figure 5.13).

At very low temperature (lower 40°C), the rate of enzyme inactivation was so slow that it is not considered. While at extremely high temperatures (higher 60°C), the enzyme activation depends on little or no transformation of the substrate. The effect of temperature should be tested by system to system of reaction condition (Ward, 1983).

Industrial scale processes with enzymes usually involves substantial reaction volumes (Tucker and Woods, 1995). This imposes limitations to the precision and time of response to temperature controlling systems. It was considered important for enzyme efficiency, especially for longer reaction times, that the set temperature should be made so that the maximum upper deviation does not allow the reaction system to rise above the observed maximum enzyme performance temperature. Typically, this can be achieved by using a set temperature 5°C lower than the enzyme maximum temperature (Godfrey and West, 1996).

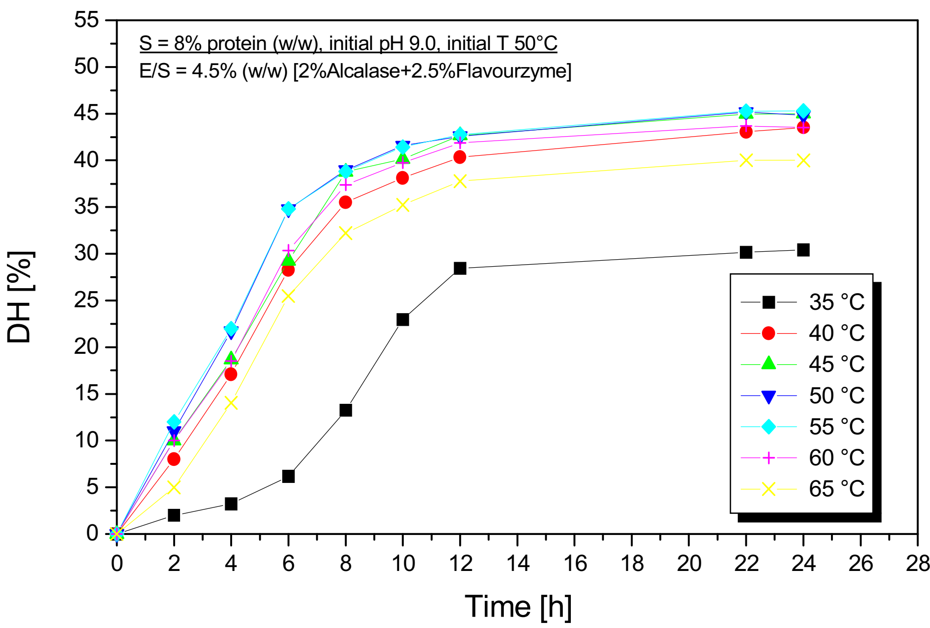



Figure 5.13 Effect of temperature on progress curve of the combination of both enzymes reaction. The hydrolysis of corn gluten meal (CGM) permeate containing 8% protein (w/w) by Flavourzyme at 2nd hydrolysis is shown at various temperatures.

Table 5.3 Some common factors affecting the effect of temperature on the reaction rate (or productivity) of an enzyme-catalyzed reaction (Godfrey and West, 1996).

| Effect | Caused by | Determined/overcome by |
|---|---|---|
| Stability of enzyme | Nature of protein | Stability/exposure experiments |
| Stability of substrate | Chemical lability | Stability/exposure experiments |
| Formation of byproducts | Inhibition | Increase [E] |
| Availability of substrate | e.g. solubility, pH | Separate experiment |
| pH functions of reacting components | Altered pK values | Heats of ionization |
| Transfer of rate-limiting functions in multi-enzyme systems | Different temperature component | Fractionate and study each enzyme separately |
| Affinity of enzyme for substrate and activator | $K_m$ | Use high [S] to ensure saturation |

#### 5.2.3.4 pH effects

Most enzymes are significantly less sensitive to changes of pH when protected by substantial substrate concentrations. The effect of pH on the affinity of the substrate can often be eliminated by using a high substrate concentration, but this may not be practical in an industrial situation (Godfrey and West, 1996). It is important that the effects of substrate concentration and temperature are also taken into consideration. This is because enzymes are often stabilized by the presence of their substrates. The optimal pH and temperature of CGM and SBM were performed by hydrolyzing with various enzymes which have discussed in Chapter 4.2. These data can be estimated the available pH value of an enzyme in the hydrolysis process.

Otherwise, it is also worth considering that many industrial enzyme reactions are not run at fixed pH value, but slowly drift from a start pH to a terminal pH (Novo Nordisk, B 841a-GB). Many processes do not have adequate pH control, and the pH may fluctuate around the required value (Reed. 1975).

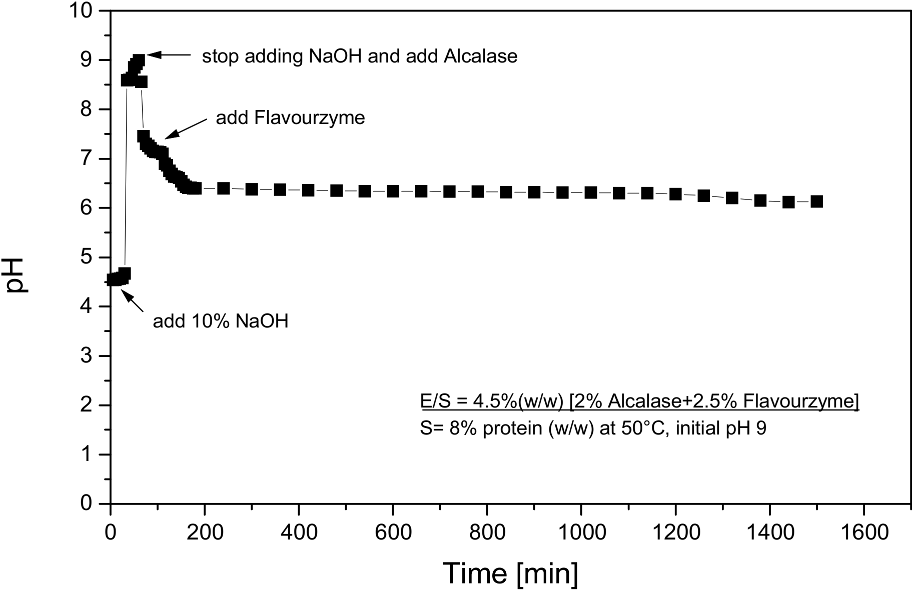


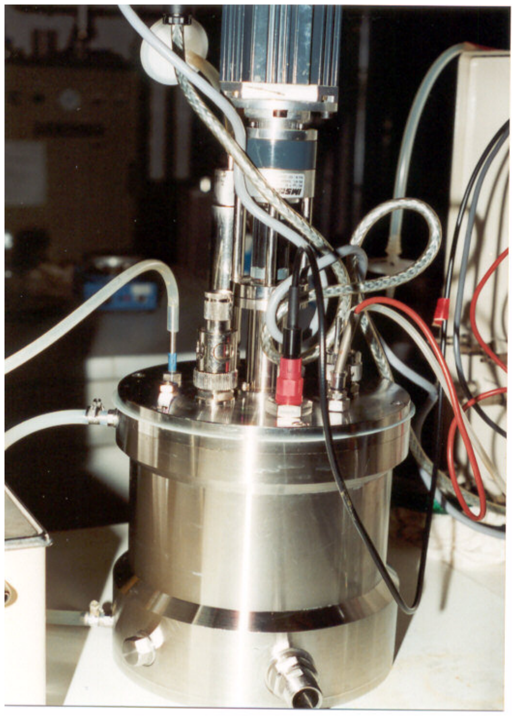

Figure 5.14 The pH control on the hydrolysis reaction of corn gluten meal (CGM) by the combination of both enzyme, no pH adjustment during hydrolysis.

In this work, the production of amino acids by enzymatic hydrolysis was operated without pH adjustment during hydrolysis. In Figure 5.14, after adding Alcalase , the pH value of reaction mixture was left to drift slowly down from initial pH 9. For 30 min the pH value will dropped

to around 7, which is the optimal pH value for adding Flavourzyme. After 24 h of hydrolysis the pH value dropped to around 6. This was still in the optimal pH range of both enzymes (Figure 5.14). The products can be obtained a reduced salt concentration by this procedure because no need for pH adjustment by adding NaOH during hydrolysis is necessary. In this cases, the data of optimum pH should be determined under controlled conditions. However, the effect of pH on stability, especially the combined interactive effect of pH and temperature, must be considered during the process.

### 5.2.4 Reproduction of enzymatic hydrolysis in 4L batch reactor

CGM and SBM were hydrolyzed by a combination of both enzymes between endopeptidase and exopeptidase in 4 L batch reactor with the following hydrolysis parameters:



S = 8 % protein (N × 6.25) (w/w)
E/S = 4.5 % (w/w) [2% endoprotease and 2.5% exopeptidase]
pH = 9.0 for CGM, 8.5 for SBM
T = 50°C
agitation = 400 rpm for CGM, 300 rpm for SBM
analysis = HPLC method

Figure 5.15 4 L batch reactor

#### 5.2.4.1 Effect of different protein substrates

Vegetable proteins such as corn gluten meal (CGM) and soybean meal (SBM) as protein substrates were tested. These substrates were a by-product (see Chapter 2.1.2) and of 8%

protein (w/w) were hydrolyzed with the combination of both enzymes, Alcalase and Flavourzyme. The dosages of the enzymes were 2% and 2.5% E/S (w/w), respectively. The conditions were at 50°C, initial pH 9 and 300-400 rpm.

The effect of different proteins on the hydrolysis of CGM and SBM is shown in Figure 5.16. It appears that the different rates of initial reaction of different substrates were defined by other factors such as the tertiary structure and the solubility of substrate.

In case of SBM, a soluble substrate, was mostly covered by catalyze enzyme. Thus, the initial reaction rate of SBM was higher than CGM, an insoluble substrate. However, the progress curve of extensive hydrolysis of CGM and SBM obtained 45% and 20% DH after 24 h of hydrolysis. These results indicate that later during the hydrolysis most substrate has been degraded and has lost its original structure. The hydrolysis rate can be related to the content of susceptible peptide bonds and free amino acids.

It was clear that enzymatic hydrolysis of CGM with the combination of both enzymes gave more amount of amino acids than using only endo or exopeptidase (Figure 5.16). Whereas, for SBM did not reach significantly % DH. SBM was possibly mixed with some substance that inhibit enzyme activity during hydrolysis.

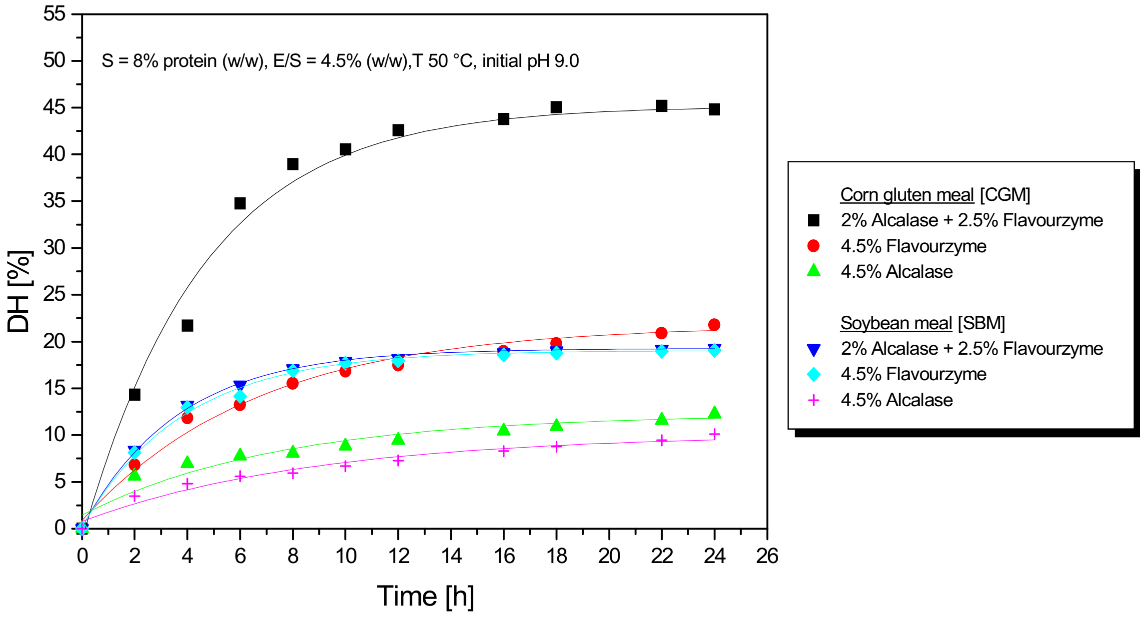



Figure 5.16 Progress curve of hydrolysis of CGM and SBM with the combination of both enzymes [2% Alcalase and 2.5% Flavourzyme] based on weight percent protein.

Obviously, the amino acid composition plays the potential protein substrate on the hydrolysis process. Based on the amino acid composition the estimation of the total peptide bonds cleaved ($h_{tot}$) led to calculate truly DH%. Normally, the limiting amino acid in cereals was lysine, without exception and lack of tryptophan in corn. The limiting amino acid in soybean meal was methionine. Figure 5.17 compares qualitatively the composition of amino acids between CGM and SBM after 24 h hydrolysis. It appears that CGM gave higher amount of amino acids than SBM, except Asp and Glu. While, CGM obtained very high Gln, Ala and Leu but Trp was very low in both raw materials.

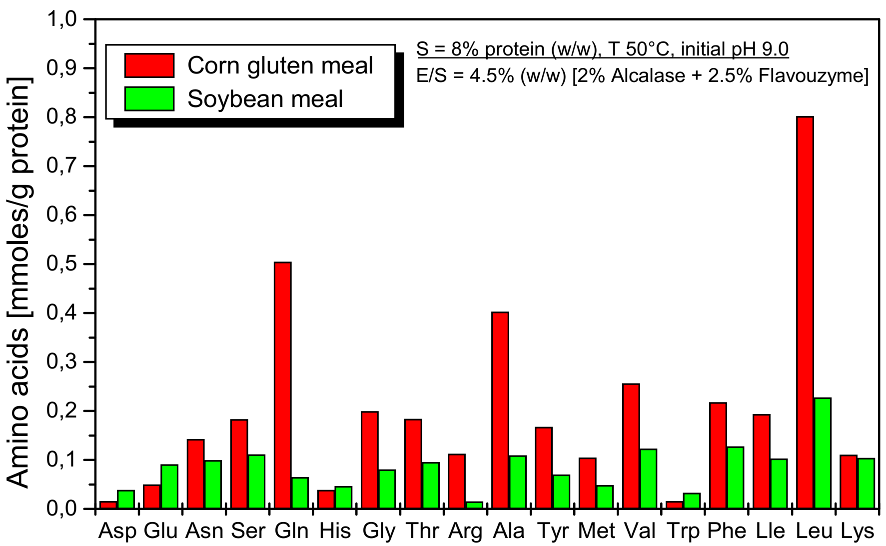



Figure 5.17 Amino acid compositions of CGM and SBM were hydrolyzed with the combination of both enzymes [2% Alcalase and 2.5% Flavourzyme] for 24 h.

#### 5.2.4.2 Effect of different endopeptidases on CGM

The information on the initial screening of endopeptidases are shown in Chapter 4. Using the endopeptidases such as Alcalase 2,4, Novo Pro-D and Novozyme FM, were obtained the highest activity by endopeptidase assay method (see Chapter 4.1.1). Their activity were pretty much the same but their cost were different. These enzymes hydrolyzed CGM at 8% protein (w/w) in the hydrolysis parameters of 4 L batch reactor. The endopeptidases mentioned were always used in protein hydrolysis, but occasionally the endopeptidases were combined with exopeptidases in order to achieve a more thorough degradation (Ward, 1983).

In this work, the different endopeptidases were used in the combination of both enzymes which hydrolyzed CGM. While, Flavourzyme was used a fixed exopeptidase. The enzyme dosage of 4.5% (w/w) [2% endopeptidase and 2.5% exopeptidase] was used.

Reaction progress curve of the hydrolysis with different endopeptidases in the combinations of both enzymes are illustrated in Figure 5.18. The whole initial velocity were linearly but progress curve of the hydrolysis declined. There could be several reasons for this behavior. **First**, severe products inhibitor may occur. The concentration of product formed by the reaction was too high to form a ternary ESP complex, which undergoes catalysis at a lower rate than the binary ES complex (Copeland, 2000). Hence, at early time the rate of product formation corresponded to the uninhibited velocity of the enzymatic reaction, but after 2 h the velocity changes to that reflective of the ESP complex. A **second** cause of burst kinetic is a time dependent conformational change of enzyme structure caused by substrate binding (Hardwick and Glatz, 1989). Herein, the enzymes were present in a highly active form but converted to a less active conformation upon formation of the ES complex. **Third**, the overall reaction rate may be limited by a slow release of the product from the EP complex (Reed, 1975). The final, and perhaps the most common, cause was rapid reaction of the enzyme with substrate to form a covalent intermediate, which undergoes slower steady state decomposition to products, like the serine protease is an for example (Copeland, 2000).

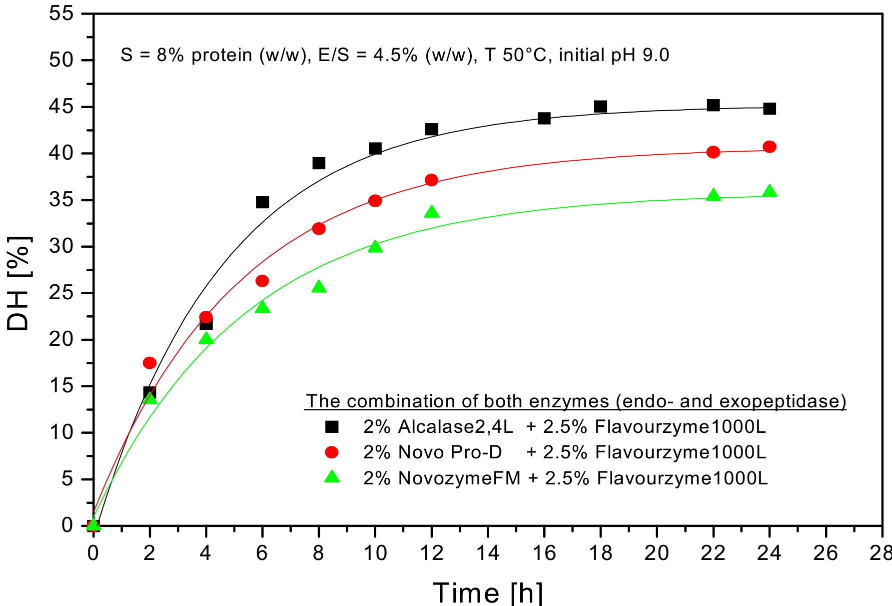



Figure 5.18 Progress curve of hydrolysis of CGM with different endopeptidases of the combination of both enzymes [2% endopeptidase and 2.5% exopeptidase].

Alcalase 2,4, Novo Pro-D and Novozyme FM belong to serine protease groups. These obtained 45, 40 and 35% DH, respectively after 24 h hydrolysis (Figure 5.18). The comparison of the price with activity of enzymes indicates that Alcalase has the highest activity with its price is cheaper than Novo Pro-D around 5 €/kg (see Chapter 4.1). Alcalase, however, is more expensive than Novozyme FM around 2 €/kg for demand very large volume. In addition, the composition of amino acids that were obtained by hydrolyzing with different endopeptidases were of the same quality. Based on these studies, Alcalase 2,4L was selected because it possessed the best combination of high activity, stability and cost effective.

#### 5.2.4.3 Effect of different exopeptidases on CGM

The exopeptidase of interest, Flavourzyme was often used in the laboratory to achieve thorough hydrolysis of protein but it is too costly for industrial use. In this case, the search for cheaper exopeptidases was necessary. It should be useful for various raw materials and must be cost effective for application in an industrial scale.

The different exopeptidases such as Flavourzyme, Kojizyme and Corolase LAP were all endo-and exopeptidase complexes (aminopeptidase and carboxypeptidase). CGM was hydrolyzed with the combination of these exopeptidases and Alcalase which was used as fixed endopeptidase. A typical dosage of the combination of both enzymes was 4.5% E/S (w/w) [2% Alcalase and 2.5% exopeptidase (w/w)]. These reactions were determined at their optimum pH and temperature of each enzyme (see Chapter 4.2).

Figure 5.19 shows the progress curve of hydrolysis for Flavourzyme in the combination of both enzymes, which resulted in the highest of hydrolysis rate but its price was costly 38.35 €/kg for large volume (see Chapter 4.2). While the economic price of Corolase LAP was 17.13 €/kg but obtained the lowest hydrolysis rate, although its dosage was increased to 5% E/S (w/w) in the reaction mixture. Corolase LAP should not be considered in the hydrolysis process. It is not worthy to produce amino acids by using Corolase LAP (see chapter 7).

Alternatively, the hydrolysis reaction of Kojizyme was higher than Corolase LAP but still lower than Flavorzyme around 10% DH after 8 h hydrolysis. Kojizyme, however is low priced at 23.26 €/kg. It appears that Kojizyme is more cost effective than Flavourzyme (see Chapter 7). Thus, Kojizyme can be used when Flavourzyme is limited.

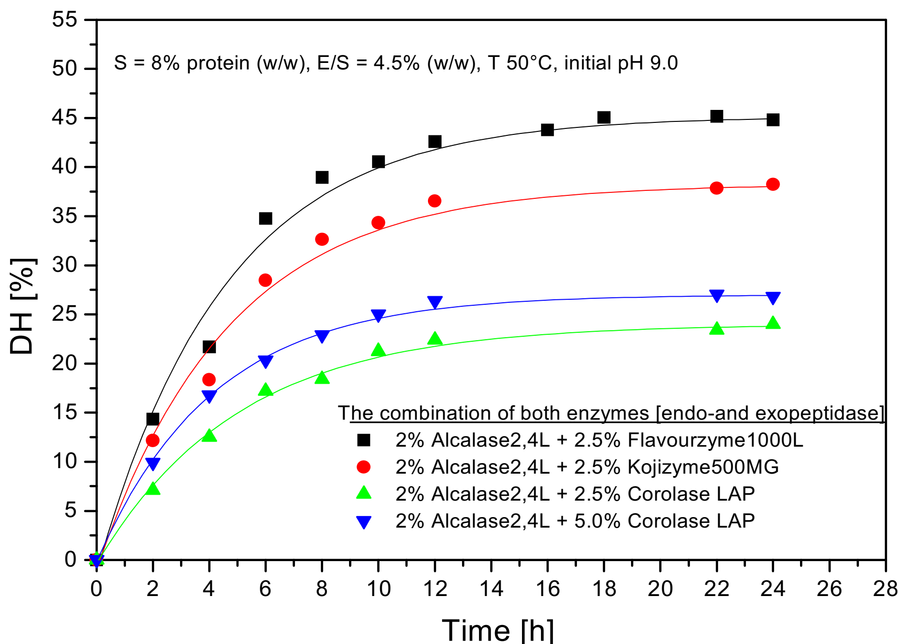



Figure 5.19 Progress curve of hydrolysis of corn gluten meal (CGM) with different combination of exopeptidases (2% Alcalase 2,4L and 2.5% exopeptidase).

#### 5.2.4.4 Effect of different endo/exopeptidases on SBM

SBM obtained DH% lower than CGM (see 5.3.1). Alternatively, the enzymatic hydrolysis of SBM should be determined. Another interesting enzyme should be found for the production of amino acids by hydrolyzing SBM.

Experimentally, SBM at 8% protein (w/w) was hydrolyzed with the different combination of enzymes (4.5% E/S, proportional to 2% endopeptidase and 2.5% exopeptidase). The different endopeptidases were Alcalase 2,4 and Novo-Pro-D and the different exopeptidases were Flavourzyme and Kojizyme. These enzymes were combined to determine SBM.

Figure 5.20 shows the progress curve of hydrolysis of SBM. The curve is similar in shape with CGM using the same typical enzymes but the reaction rate was lower around 15-25% DH at the overall reaction. Substrate limitation might be the reason for this. The conclusion which can be drawn from the above experiment was that CGM should be the selected substrate for hydrolysis with Alcalase and Flavourzyme with consideration for concerning the amino acids compositions and its state of degradation. Unfortunately, SBM was not a possible substrate in the production of amino acids even it was an enormous protein sources as well as region availability.

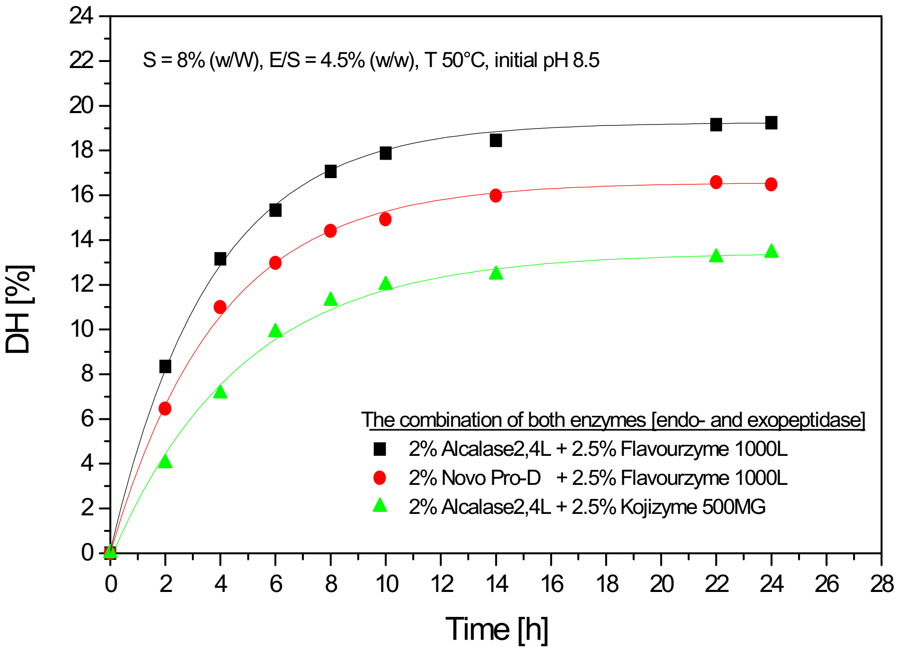



Figure 5.20 Progress curve of hydrolysis of soybean meal (SBM) with different endopeptidases and exopeptidases (2% endopeptidaes and 2.5% exopeptidase).

#### 5.2.4.5 Effect of pretreatment on CGM

The protein substrate should be in a physical form which makes it accessible for enzyme degradation. Refined and processed vegetable proteins, such as soybean meal and corn gluten meal are already in such a form (see Chapter 3.1). Heat treatment of the protein substrate prior to hydrolysis will denature the native globular protein, making it more accessible for enzyme degradation, and help to prevent microbiological spoilage during hydrolysis, which extend to many hours. For this reason, pretreatment of CGM was determined because its solubility is less than SBM. The reaction mixture of CGM was adjusted to pH 9 and then heated to 80°C for 2 h prior to hydrolysis.

It was clear that Alcalase plus Flavourzyme apparently cannot completely hydrolyze CGM proteins. As pretreatment of CGM improved hydrolysis rate, which obtained hydrolysis rate higher than non-pretreatment around 5% DH after 24 h hydrolysis (Figure 5.21). This pretreatment affected also the other enzymes such Novo Pro-D plus Flavourzyme, which obtained around 5% DH increasingly.

The effect of pre-denaturation on hydrolysis of native globular proteins was derived using one by one versus zipper reaction models (see 5.2.1.2). If the hydrolysis were not of the one by

one type, large peptides would also be present, and consequently the yields and fluxes obtained would be lower (Adler-Nissen, 1986).

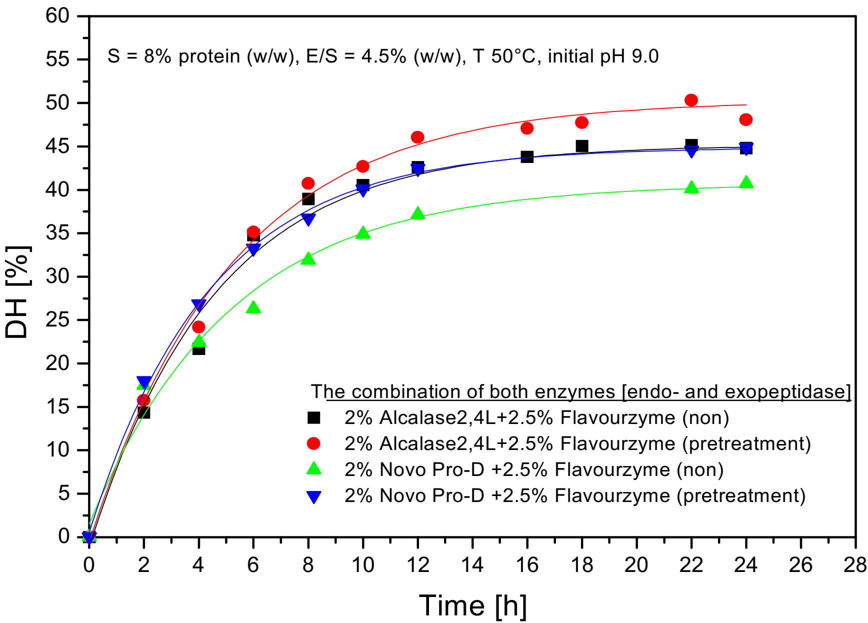



Figure 5.21 Effect of pretreatment on corn gluten meal (CGM) prior hydrolysis with different combination of enzymes (2% endopeptidase and 2.5% exopeptidase).

### 5.2.4.6 Effect of enzyme grades on CGM

Enzymatic hydMany enzymes were manufactured in different purity grades and this was reflected in their cost. In general, there are four enzyme grades; analytical, pharmaceutical, food, and technical (US Food and Drug, 1993).

The proteases in this work were supplied by Novo Nordisk. The technical grade enzyme was marketed cheaper than the food grade enzyme around 4.60 €/kg for Alcalase (see Chapter 4.1). The different endopeptidases between food grade and technical grade were combined with a fixed exopeptidase as Flavourzyme (food grade). The enzyme dosage of this combination was 4.5% E/S (w/w) [2% endopeptidase and 2.5% Flavourzyme] which hydrolyzed CGM at 8% protein (w/w).

Figure 5.22 shows that the combination of enzyme for food grade obtained hydrolysis rate higher than technical grade around 10% DH after 10 h of hydrolysis. Food grade enzyme is a crude enzyme, which may have substances which are either suitable activators within or/and a tolerance of the ingredient in substrate raw materials.

In addition, food grade enzymes require a considerable financial investment in terms of toxicological testing. That why their cost are higher than technical grade in the same enzymes even lower purity. However, the law requires that food grade enzymes must be used for food applications and should be used for feed applications, no matter what the process are like (see Chapter 2.3.4).

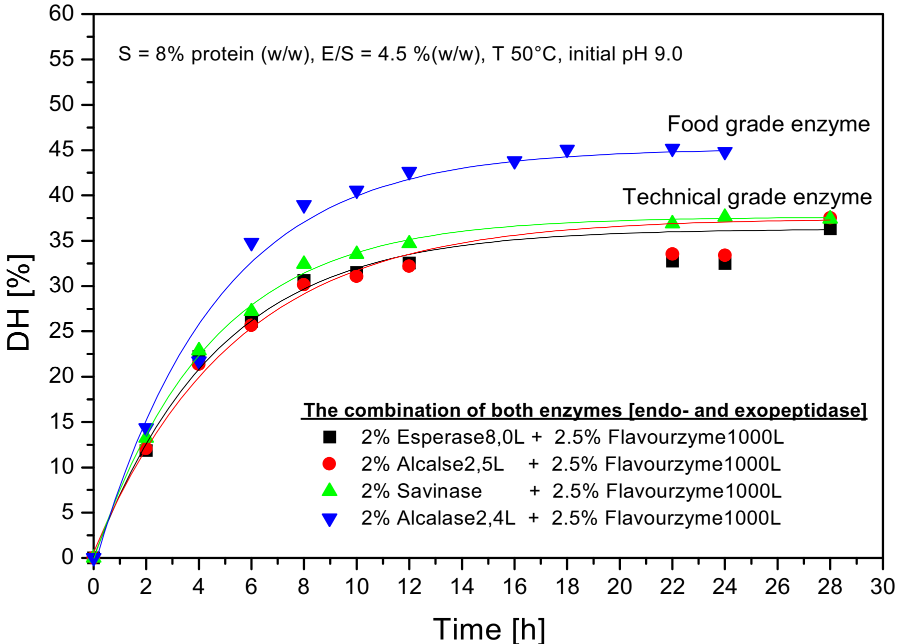



Figure 5.22 Effect of food grade enzyme and technical grade enzymes on corn gluten meal (CGM) with different combination of enzymes (2% endopeptidase plus 2.5% exopeptidase).

### 5.2.5 Summary

In applied emzymology, it is not too concerned with the mechanism of the effects but expressed in a quantitative and practically meaningful way. Data are usually expressed graphically in the form of maximum reaction rate as a function of the variable, in order to interpret the optima values easily. Kinetic considerations were applied for a qualitative description of curve shape and the composition of the resulting amino acids and a quantitative assessment of the effect of changing the hydrolysis conditions, for example, temperature or enzyme concentration.

At very low substrate concentrations, the $v$ versus [S] curve is essentially linear; that is, the velocity (for all practical purposes) is directly proportional to the substrate concentration. This is the region of *first-order kinetics*. At very high substrate concentrations, the velocity is

essentially independent of the substrate concentration. This is the region of *zero-order kinetics*. The shape of reaction curve often followed the integrated Michaelis-Menten equation. The effect of pre-denaturation prior to the hydrolysis reaction was also derived using one by one versus zipper models. One by one type is slow initial steps but rapid decomposition of primary products. Zipper type models is rapid initial steps but slower decomposition of primary and secondary products. Throughout the progress curve, the velocity was changing as the substrate concentration available to the enzyme continues to diminish. The cost effects of an enzyme-catalyzed process was achieved by overall cheaper processing, with a higher product yield.

The protein hydrolysis rate were carried out using in a 0.1 L reactor and were reproduced in a 4 L batch reactor. The hydrolysis reaction of a well-stirred reactor was scale independent behavior. The combination of both enzymes [Alcalase and Flavourzyme] was selected for use in the hydrolysis process. The velocities were measured as the slopes of the plots of amino acid concentrations [P] versus time. The zero-and first-order kinetics suggested that amino acids concentration were time dependent. The kinetics were determined by substrate saturation at 8% protein for CGM and 10% for SBM into the reaction mixture. Thereby, the conversion rate expressed the increase amino acids per unit of time that was proportional to the mass ratio of enzyme to substrate. In addition, this ratio also forms the basis for assessing the contribution to the variable cost from using the enzyme. However, the enzyme concentrations around 3.7-5.0% E/S (w/w) [0.5-2.0% Alcalase and 1.0-2.5% Flavourzyme] were recommended to use in the hydrolysis process.

The effect of temperature on the reaction rate and overall product yield of an enzyme reaction can be due to several factors, for example, natural proteins, by-product inhibition and chemical lability. The initial velocity of hydrolysis curves were linear but progress curve decline. Hence, at early time the rate of product formation corresponded to the uninhibited velocity of the enzymatic reaction, but after 2 h the velocity changed to that reflective of the ESP complex, which undergoes catalysis at a lower rate than the binary ES complex.

The function of the statutory specifications of enzyme purity to ensure that the correct grade must be used for the correct purpose.

# 6 Extremozymes

## 6.1 Introduction

The majority of enzymes used to date have been obtained from mesophilic organisms. The application of these enzyme is restricted because of their limited stability to extremes of temperature, pH, ionic strength, etc. Extremophiles are a source of enzymes (extremozymes) with extreme stability, and the application of these enzymes as biocatalysts is attractive because they are stable and active under extreme conditions that were regarded. Furthermore, it is clear that some extremophiles have novel metabolic pathway and so might serve as a source of enzymes with novel activities and applications (Hough, 1999).

Up to now, thermostable polymer-degrading cellulases, xylanases, amylases and proteases as well as sugar-converting isomerases are good examples of enzymes that either have potential or already found their application in industry (Krahe et at., 1996). Extremozyme can potentially eliminate the need for working on extreme conditions, thereby increasing efficiency and reducing costs. However, the diversity of organisms in extreme environments is far greater than was initially suspected. As the enzymes, transforming a newly isolated extremozyme into an available product for industry needs several years (Madigan and Marrs, 1997).

In this work, extremozymes from genetic engineering and the isolated strain were characterized by hydrolyzing corn gluten meal (CGM). Of equal importance, enzyme engineering and directed evolution provide approaches to improve enzyme stability and altered specificity.

## 6.2 Extremophlies

Extremophiles are organisms that require extreme environments for growth. Extremophiles are therefore organisms that are "fond of" or "love" (-phile) extreme environments (Cavicchioli, 2000) including high temperature (thermophiles), pH, pressure and salt concentration, or low temperature, pH, nutrient concentration, or water availability. In addition, extremophiles may be found in environments with a combination of extreme conditions such as high temperature and high acidity or high pressure and low temperature.

The majority of extremophiles identified to date are members of the Archaea (Woese, et al., 1990). Although, mesophilic Archaea have been identified, the majority of archaeal species have been detected in extreme environments. Extremophillic bacteria have also been

identified, and it is interesting to note that they are among the most primitive bacteria, as suggested by their location close to the root of the universal phylogenetic tree (Figure 6.1).

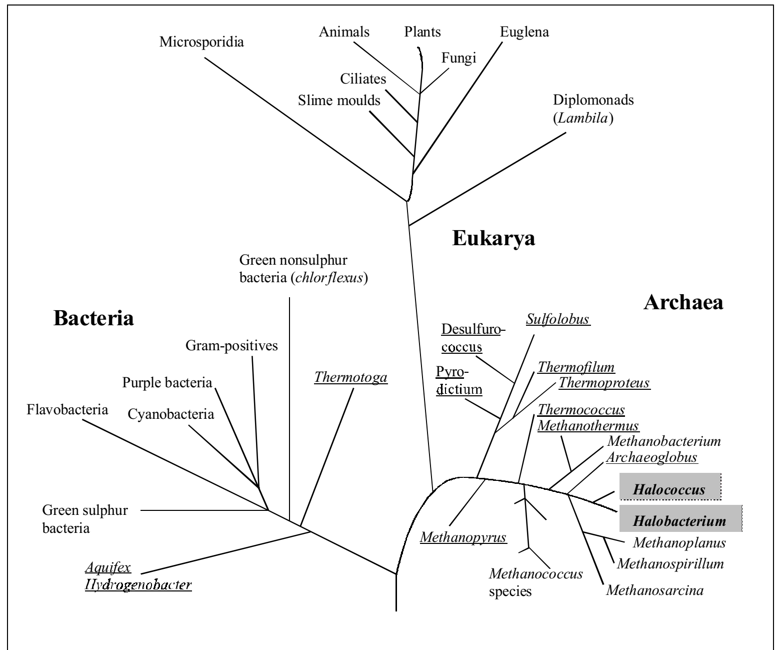



Figure 6.1 Phylogenic tree of thermophilic organisms. Branches representing the three domains (Archaea, Bacteria, Eukarya) are indicated. Thermophilic and hyperthermophilic species are underlined and halophilic species are shaded. Branching order and branch lengths are based upon 16/18S rDNA sequence comparisons. The tree is a modified version from Blöchl et al (1995). Reproduced with permission from (Danson and Hough, 1998).

The presently known upper temperature limits for archaeal, bacterial and eukaryotic microorganisms are 113°C, 95°C and 62°C respectively. It should be noted that archaea are also found in a broad range of "non-extreme" marine and soil environments (Hough, 1999).

Microorganisms that are adapted to grow optimally at high temperatures (60-108°C) have been isolated from high temperature terrestrial and marine habitats. The most common bio-topes are volcanically such as solfataric fields, neutral hot springs, and submarine hot vents (Stetter, 1996). A keratin degrading thermophilic bacterium (*Fervidobacterium pennavorans*) was isolated from hot springs of the Azores Island and is able to convert chicken feather completely to amino acids and peptides at 80°C within 48 h (Rehm and Reed, 2001).

Extreme thermophiles, which grow optimally between 60°C and 80°C are widely distributed among the genera *Bacillus, Clostridium, Thermoanaerobacter, Thermus, Fervidobacterium,*

*Thermotoga and Aquifex*. Most of the hyperthermophiles, grow optimally between 80°C and 108°C (Stetter, 1998), which have been studied on isolation, classification and properties.

The developments in the field have arisen due to the isolation of extremophiles from environments previously considered impossible for sustaining biological life. In addition, the novel cellular components and pathways identified in extremophiles have provided a burgeoning new biotechnology industry.

The classical route to microbial enzyme production, involving isolation of the organism as a pure culture followed by purification of the enzyme either from its natural host or by cloning and expression of the corresponding gene has been used to obtain extremozymes (Connaris et al., 1998; Kim et al., 1998).

### 6.3 Extremozyme production

By applying modern technologies such as molecular biology and genetic engineering, it has been possible to produce enzymes from extremophiles at high concentration and analyze their structure and function in more detail. As the cloning and expression of these enzymes in mesophilic hosts opens new possibilities for novel industrial processes.

Attempts to enhance the stability of mesophilic enzymes or to manipulate the specificity of extremozymes by directed evolution depend on the availability of the appropriate cloned genes and expression systems.

The viability of processes based on the use of extremozymes is dependent on the availability of enzymes in sufficient quantity for practical application. This immediately raises problems if the enzyme is isolated directly from the source organism or for *in situ* reactions involving whole cells. The conditions required for the growth of extremophiles include temperatures above 80°C (hyperthermophiles), often in an anaerobic environment, and media of extreme pH (acidophiles and alkaliphiles). These conditions are incompatible with standard industrial fermentation and the downstream processing plant. Most studies on extremozymes have focused on thermophilic enzymes. Similarly, the cultivation methods are related to the problems of growing thermophilic and hyperthermophilic microorganisms.

Because the problems presented by large-scale cultures of extremophiles and subsequent purification of extremozymes, most applications rely on the expression of the corresponding gene in a mesophilic host. This approach avoids problems arising from the need to grow extremophiles, but it introduces further complications resulting from the expression of genes

encoding extremozymes in a non-native, mesophilic environment. It is now generally accepted that genes from thermophiles and hyperthermophiles can be expressed successfully in a mesophilic host such as *E. coli*, although difficulties might be encountered when the expressed enzyme requires specific cofactors or metal ions that the host does not utilize (Hough, 1999). Expression normally yields a soluble product that has similar activity to the native material when assayed at an appropriate temperature (Connaris et al., 1998). Furthermore, heat precipitation of host cell protein provides a simple initial stage in purification of the expression product. On occasions, the expressed product is assembled in a slightly different oligomeric state from the native material (Hess et al., 1998), although this has little impact on the stability or catalytic properties of the enzyme.

Although, there are no reports in the literature to date, that gene encoding extracellular extremozymes stable at high or low pH are expressed in a mesophilic hosts (Hough, 1999). Expression of such enzymes in a mesophilic host such as *E. coli* would not allow the proteins to fold in a native-like environment and therefore problems are likely to arise as a result of insolubility or inactivity.

## 6.4 Characterization of extremozymes on corn gluten meal (CGM)

Several thermophilic microorganisms that grow in state at 150°C, pH 0.7-11, for example; *Fervidobacterium islandicum, F. nodosum, F. pennavorans, F. pennavorans* Ven5, *T. maritima*, *T. neapolitana*, *T. thermarum*, *Thermosipho africanus* (Krahe et at., 1996). As the activity of proteases from these microorganism have been determined to define for the potential fragments of new proteolytic enzyme by genetic engineering. The gene fragments of proteases have been cloned in *E. coli* which is a mesophilic host and expression of the recombinant proteases work on production in this mesophilic host.

In this work, the department of microbiology at the university of Hamburg-Harburg supplied two proteolytic enzymes from extremophilic microorganisms, *Thermotoga maritima* and *Thermoanaerobacter keratinophilus sp. nov*. As both extremozymes have been determined the activity of endopeptidase assays by using Azocasein as substrate. The enzymes were assayed the activity of endopeptidase again by using casein as substrate before they were allowed expression of enzyme activity by hydrolyzing corn gluten meal (CGM).

### 6.4.1 The recombinant protease from *Thermotoga maritima*

The heterologous expression of active carboxy-terminal protease from hyperthermophilic archaeon *Thermotoga maritima* (Figure 6.2) in the mesophilic hosts *Escherichia coli* LMG194 was resulted in the formation of recombinant proteases (Table 6.1) . This extremozyme is an endopeptidase. It splits between two Alanines in unpolar regions around 30 amino acids from the carboxy-terminus.

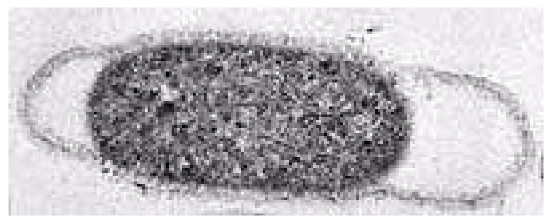


Figure 6.2 *Thermotoga maritima*, another heat-loving bacterium, or thermophile

Table 6.1 Summary of recombinant *E. coli* strains containing genes for thermoactive proteases from ITU Hamburg-Harburg

| Source | Carboxy-terminal protease | Serine type protease | Processing protease |
|---|---|---|---|
| *Thermotoga maritima* | → active clone (in vector pBadgIII)<br>→ the partially purified recombinant protease | active clone (in vector pET 15b) | active clone (in vector pET 15b) |

After the disruption of recombinant cells with ultrasonic treatment, extra/intracellular crude enzyme has been purified partially by heat precipitation at 90°C for 10 min. The activity of crude enzyme has been determined with 0.7% Azocasein as substrate in universal buffer pH 8. The absorption of supernatant has been measured at 450 nm (the activity was 4 Unit/ml). The protein content was 400 µg/ml (the specific activity was 10 Unit/mg protein). It appeared that the optimum pH and temperature of the recombinant carboxy-terminal protease were pH 9.0 and 80°C. These result have been tested by the Department of Microbiology, University of Hamburg-Harburg.

In this work, this enzyme (the carboxy-terminal protease from *Thermotoga maritima*) was provided for degradation of proteins such as casein and corn gluten meal (CGM). First, the activity of enzyme was tested using casein as substrate by endopeptidase assay methods (see

Annex 9.5). Therefore, the activity of enzyme was compared to the activity of other commercial enzymes (see Chapter 4.1.1). The activity of this recombinant protease was 3 $U^{cas}$/ml and the specific activity was 4.38 $U^{cas}$/mg protein by Bradford assay (Annex 9.1). The optimum pH and temperature were determined by hydrolyzing CGM [1% protein (w/w)] as substrate (2 ml reaction volume, 700 rpm for 24 h of hydrolysis).

The data in Figure 6.3 and Figure 6.4 show the amount of amino acids were measured by HPLC analysis and the soluble protein by the Braford assay (see Annex 9.1). As the soluble protein represents the affected chemical and physical properties as pH and temperature. The solubility of protein increased at high pH value (above 10) and temperature (above 70°C). But no effect of pH and temperature on the activity of recombinant protease for CGM as substrate because the amount of amino acids were obtained very low. The data were compared to those without enzyme hydrolysis in Figure 6.5. There may be some substances in CGM that inhibited enzyme activity. The hyperthermophiles can be expressed successfully in a mesophilic host such as *E. coli*, but similar difficulties might be encountered when the expressed enzymes requires specific cofactors or metal ions that the host does not utilize. On the other hand, expression of enzymes in a mesophilic host would not allow the proteins to fold in a native-like environment and therefore problems are likely to arise as a result of insolubility or inactivity (Hough, 1999).

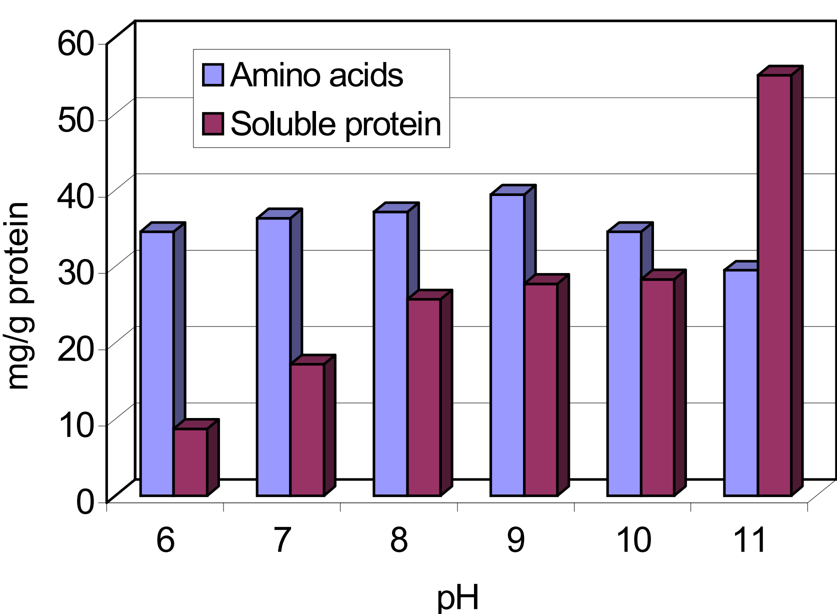



Figure 6.3 Characterization of the recombinant carboxy-terminal protease from *Thermotoga maritima*. Enzyme conc.; 30 $U^{cas}$/g protein hydrolyzed 1% protein (w/w) of corn gluten meal (CGM) at 80°C for 24 h hydrolysis.

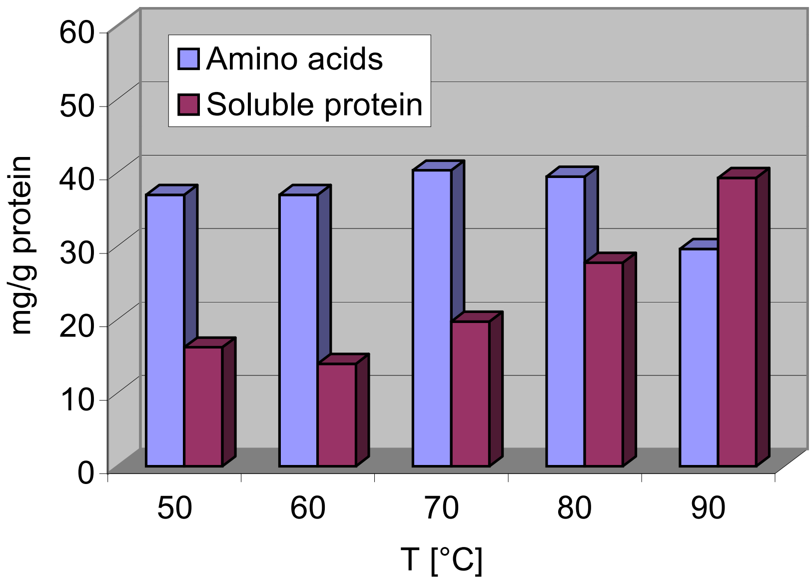



Figure 6.4 Characterization of the recombinant carboxy-terminal protease from *Thermotoga maritima*. Enzyme conc.; 30 $U^{cas}$/g protein hydrolyzed 1% protein (w/w) of corn gluten meal (CGM) at pH 9.0 for 24 h hydrolysis.

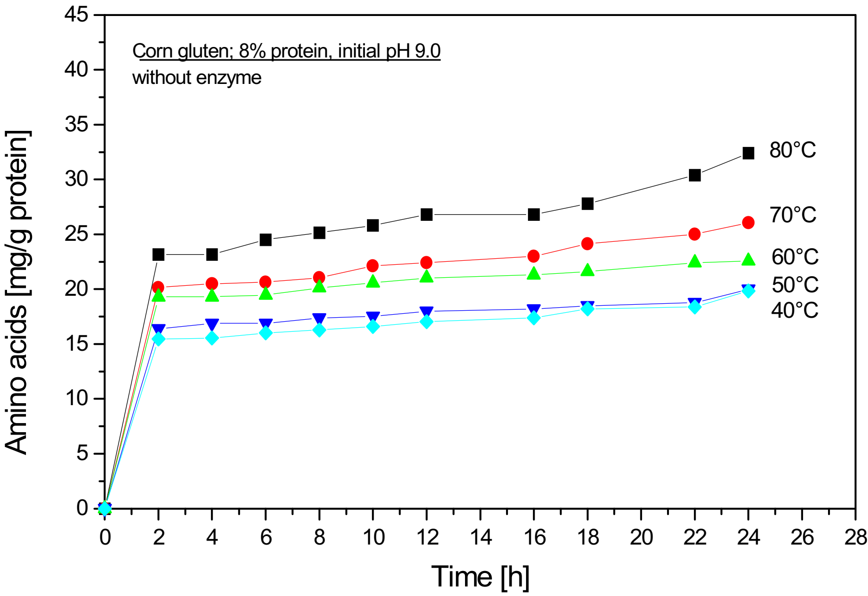



Figure 6.5 Effect of different temperatures on corn gluten meal at 8% protein (w/w) in a volume mixture 100 ml, stirred well without enzyme (control reaction).

### 6.4.2 The isolated protease from *Thermoanaerobacter keratinophilus sp. nov*.

In the screening program of thermophilic, anaerobic microorganism, *Thermoanaerobacter keratinophilus sp. nov* has been isolated (Riessen and Antranikian, 2001). It grows at 70°C in neutral pH value and produces extracellular protease. The extracellular protease from *T. keratinophilus* has optimum temp of 85°C and optimum pH of 8.0 and is still stable at 70°C. As protease has been incubated at a temperature of 70°C over a period of 24 h without losing the proteolytic activity. $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ affect the activity of enzyme positively also. The activity of crude enzyme has been assayed with Azocasein as substrate (2 Unit/ml). The data above were given by the Department of Microbiology, at University of Hamburg-Harburg.

In this work, other substrates such as casein and CGM were used to determine this enzyme activity. This extracellular enzyme was assayed with casein as substrate by the endopeptidase assay methods (see Annex 9.6) to compare enzyme activity with the other commercial enzymes (see Chapter 4.1.1). The activity of this protease was 1 $U^{cas}$/ml and the specific activity was 3.80 $U^{cas}$/mg protein. The different concentrations of this enzyme were determined by hydrolyzing CGM [8% protein (w/w) in 100 ml reaction mixture, initial pH 8.0 and 700 rpm], after the optimum pH and temperature were characterized. The amino acids were analyzed by HPLC method.

The effect of different temperatures on the hydrolysis rate for CGM are shown in Figure 6.6. The optimum temperature was defined as 80°C if the initial velocity was considered as a criterion. However, if the progress hydrolysis was considered, the optimum temperature for the reaction were identified as 70°C. The decline in reaction progress with time (*dP/dt*) at the higher temperatures can be explained by the progressive inactivation of enzyme during incubation.

The effect of different pH values on the progress curve of hydrolysis for CGM are shown in Figure 6.7. The initial velocity of pH 7 and 8 obtained the highest rate but after 10 h of hydrolysis the velocity of pH 8 became higher than pH 7. As this protease was less active on base conditions around pH 10-12 and on acid condition around pH 6. However, the optimum pH and temperature were pH 8 and 70°C. At this optimum conditions, the hydrolysis rate of this protease was 1.76% DH after 24 h of hydrolysis. It appears that this extremozyme was lower active than Alcalase 2,4L which was a commercial endopeptidase.

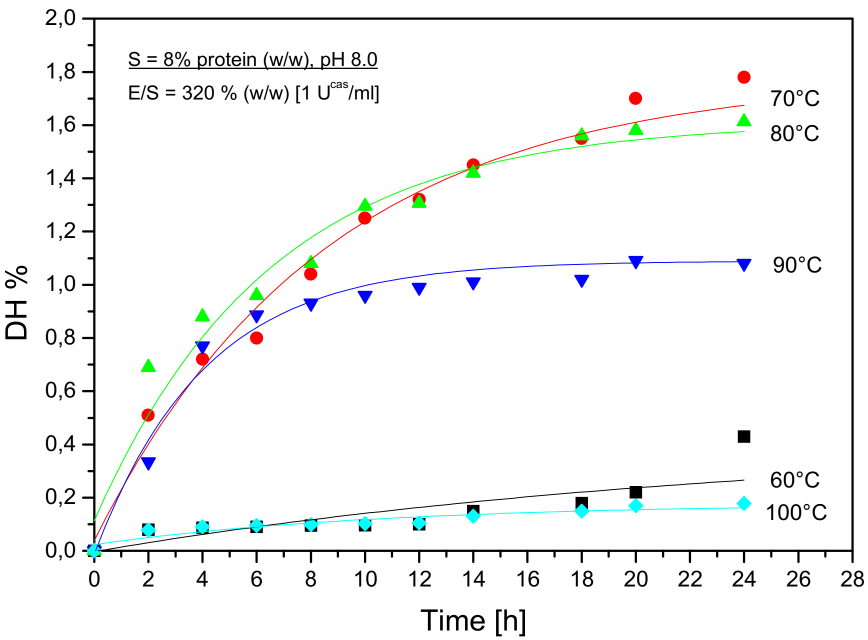



Figure 6.6 Stability and activity of extremozyme from *Thermoanaerobacter keratinophilus sp. nov.* by hydrolyzing corn gluten meal (CGM) at 8% protein (w/w), enzyme conc. 320% E/S (w/w) and pH 8.0.

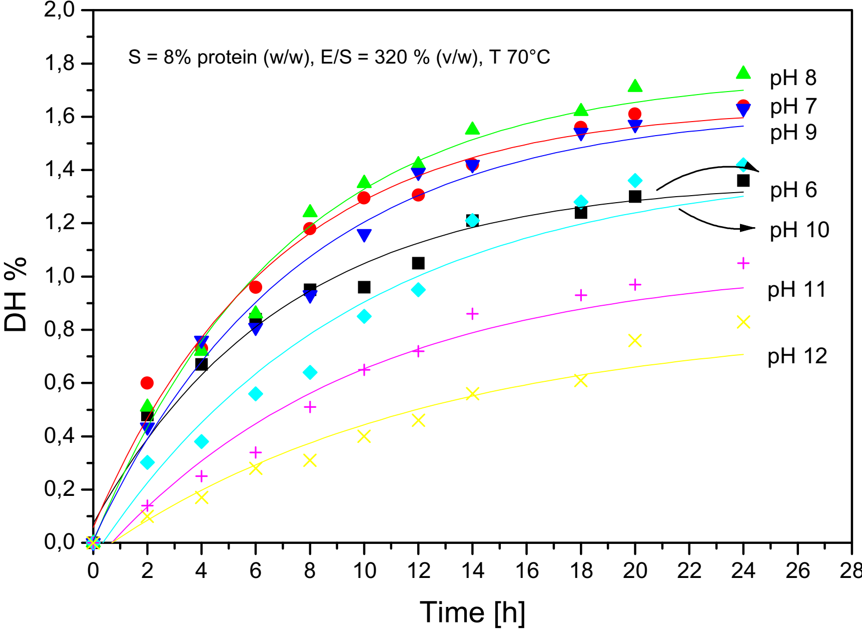



Figure 6.7 Effect of different pH on progress curve of hydrolysis of extremozyme from *T. keratinophilus sp. nov.* by hydrolyzing corn gluten meal (CGM) at 8% protein (w/w), enzyme conc. 320% E/S (w/w) and 70°C.

Figure 6.8 shows the effect of different concentrations of endopeptidase from *T. keratinophilus* on the progress curve of hydrolysis. Also these enzyme concentrations were combined with Flavourzyme which is an affective commercial exopeptidase. It appears that all the initial velocity of hydrolysis curve increased proportionally with enzyme concentration but the extent hydrolysis decline due to the inhibition of end-product. Thus, the rate of hydrolysis depends on enzyme concentration and the combination of enzymes with Flavourzyme that was more affective.

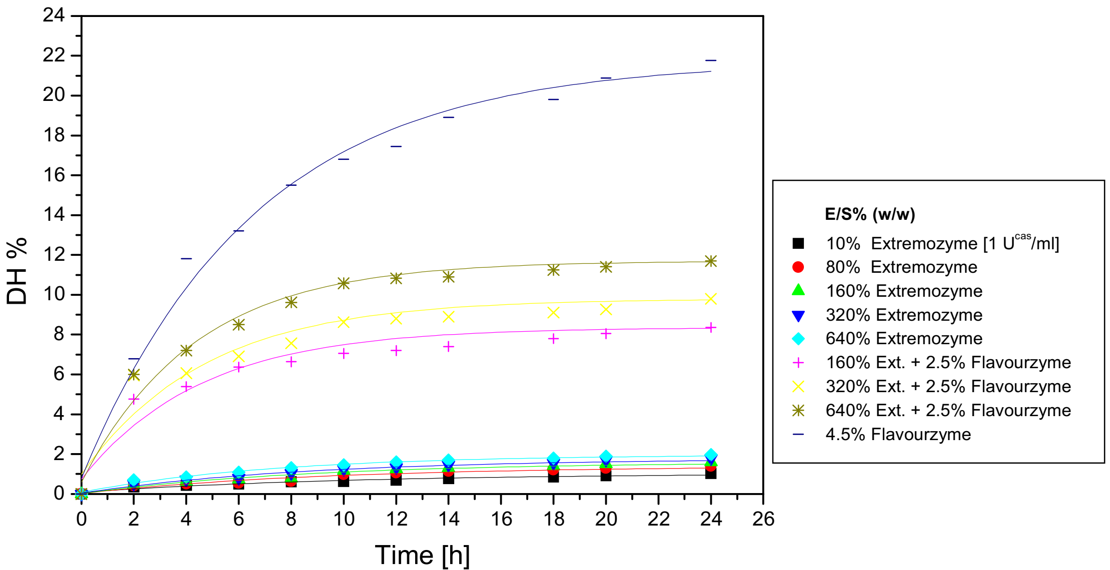



Figure 6.8 Progress curve of hydrolysis of extremozyme from *T. keratinophilus sp. nov.* acting on corn gluten meal (CGM) at 8 %protein (w/w), 70°C, initial pH 9.0.

## 6.5 Summary

The both new proteolytic extremozymes [a recombinant protease from *Thermotoga maritima* and an isolated enzyme from *Thermoanaerobacter keratinophilus*] were characterized by hydrolyzing different proteins such as casein and CGM. The viability of processes based on the use of extremozymes is dependent on the availability of enzyme in sufficient quantity for practical application. Because the problems presented by large-scale culture of extremophiles and subsequent purification of extremozymes, most applications rely on the expression of the corresponding gene in a mesophilic host. It appears that the both enzymes have very low activity after hydrolyzing on CGM to produce amino acids.

# 7 Process Economics

Economics plays an important role in process research, development and commercialization. In general development, amino acid production by enzymatic hydrolysis which begins with a process flow concept, works through process equipment sizing, materials and utility needs, costs of initial plant and of operations, and an estimate of profitability. As the relative cost importance of substrate feedstocks, equipment, utilities, and reactor vs. recovery sections will be examined, since identification of major cost areas frequently pinpoint process economic weakness and thereby indicates process improvement which would be most logical to pursue in process optimization.

In the long run, the project is expected to recover its costs and return an appropriate profit. Thus, an immediate need arises, given a process or product inception, to carry out a preliminary evaluation of economics and market. If the preliminary evaluations are promising, the development of data for final design normally follows. This development includes both market and cost refinement. The cost studies include development of a complete plant process, determination of corresponding capital and operating cost, and estimation of process profit each year over the assumed lifetime or pay-out period. These latter results would also include a simple sensitivity analysis to establish the key assumed cost and performance measures which most strongly affect the profitability calculations, since costs may change significantly before a process is realized.

The goal of this section is to calculate the cost of the input material in the production of amino acids by enzymatic hydrolysis process to produce a high valuable product. Of the twenty amino acids, only four (Phe, Tyr, Ile, Leu) were calculated primarily, although more are potentially producible. However, the hydrolysis times affected to the different amounts of amino acids were also considered.

## 7.1 Enzymatic hydrolysis costs

The economic aspects of enzymatic hydrolysis are now considered. The data of the effect of different concentrations of substrate and enzyme on hydrolysis rate from Chapter 5.2.3.1 and 5.2.3.2 were assumed for production costs (Table 7.1) which led to profitability calculation (Figure 7.1 and Figure 7.2). As the production cost of enzymatic hydrolysis for 10 tons CGM was estimated about 15,842.34 € per cycle time (with the standard conditions were of 8%

protein (w/w), 2% Alcalase and 2.5% Flavourzyme [E/S% (w/w)]). After 8 h of hydrolysis obtained profit about 5,200 € for one cycle time. The details of calculation are in Annex 9.13.

Table 7.1 Production costs of enzymatic hydrolysis at an estimated 10 tons corn gluten meal [CGM]. Conditions; substrate conc.: 8% protein (w/w), enzyme conc.: 2% Alcalase 2,4 L and 2.5% Flavourzyme 1000L [E/S% (w/w)], at 50°C, and initial pH 9.0.

| Cost factors | Amount [kg] | Unit cost [€] | Total cost [€] |
|---|---|---|---|
| Corn gluten meal | 10,000 | 0.64 | 6,391.15 |
| Alcalase 2,4 L | 124.76 | 19.94 | 2,487.76 |
| Flavourzyme 1000 L | 155.95 | 35.79 | 5,980.20 |
| NaOH | 269.00 | 0.09 | 24.76 |
| Water | 71,170.27 | 0.00028 | 19.65 |
| Machine | -- | -- | 34.86 |
| Personal | -- | -- | 903.96 |
| Σ | **81,719.96** | | **15,842.34** |

Assumptions:

1. A sized vessel is assumed: 20 $m^3$. The operating volume is 16 $m^3$ of liquid (16 tons).
2. For a production per cycle of 81,720 kg and a production per batch of 16,000 kg. Thus, the number of 20 $m^3$ vessels needed is (81,720/16,000) ≈ 5.
3. A machine cost is 46,016.27 €. Depreciation: 10% of capital (10 year straight line) is 4,602 € per year [(4,602/330 day) = 13.94 € per day].
4. With a 8 h cycle time (2 cycle per day), a vessel can provide (13.94/2) = 6.97 € per cycle. For 5 vessels are (5 × 6.97) 34.86 € per cycle.
5. Personal cost is based on manufacture payment. In case of the chemical industry at Amino GmbH, the personal cost is 13.29 € per hour.
6. 5 persons work on 5 machines in an operation, the factor of incidental expense is 1.7 that can provide ([(5 × 8 h) × 13.29] × 1.7) = 903.96 € per cycle.

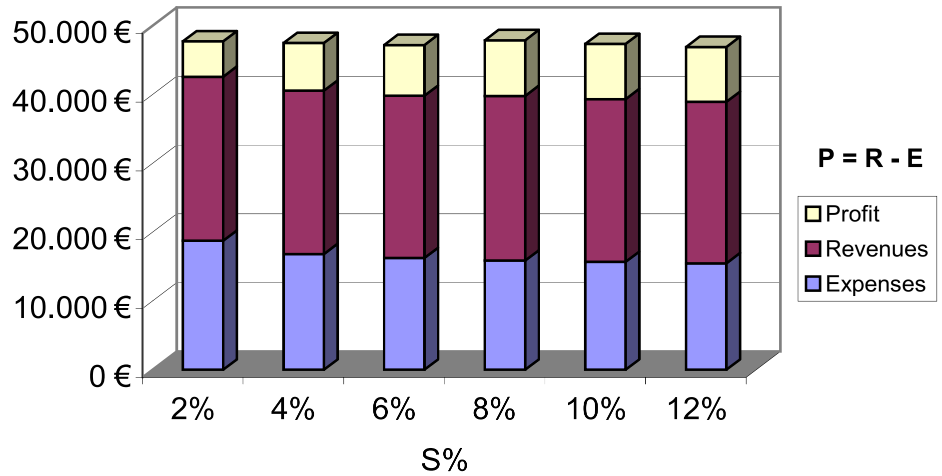



Figure 7.1 Economic calculation of different starting substrate concentrations of corn gluten meal (CGM) for 10 tons were hydrolyzed with 4.5% E/S (w/w) at 50°C and initial pH 9.0. Revenues cost were calculated only for 4 amino acids such as Tyr, Phe, Ile and Leu [18.92, 34.26, 48.57 and 21.99 €/kg respectively] (Annex 9.13).

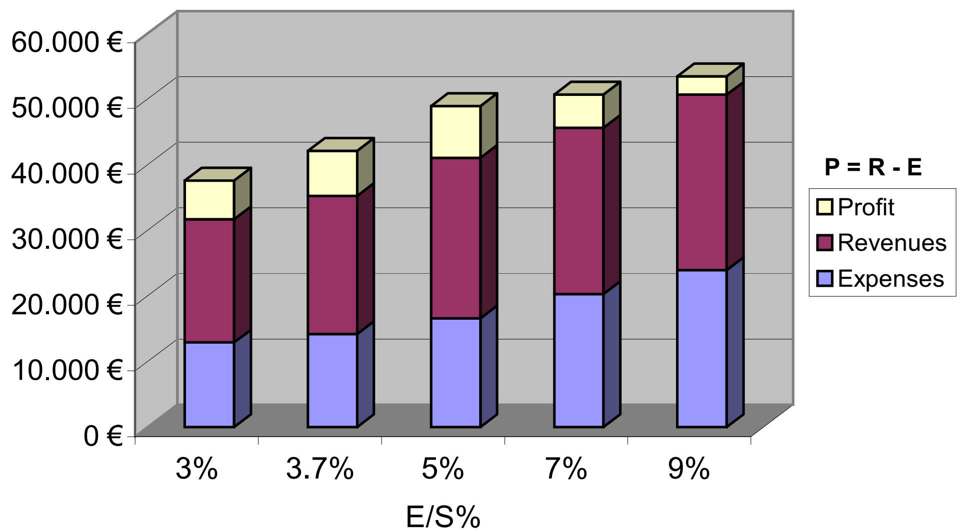



Figure 7.2 Economic calculation of different starting enzyme concentrations based on weight percent protein as substrate, hydrolyzed corn gluten meal (CGM) for 10 tons of 8 % protein (w/w) at 50°C and initial pH 9.0. Revenues cost were calculated only for 4 amino acids such as Tyr, Phe, Ile and Leu [18.92, 34.26, 48.57 and 21.99 €/kg respectively] (Annex 9.13).

The different combinations of both enzymes between endo-and exopeptidases which have different unit price and activities were taken into access. These data from Chapter 5.3.2.2 and 5.3.2.3 were used to calculate profitability. compare to each other. As the combination of both enzymes, Alcalase and Flavourzyme obtained the highest profit that was estimated about 5,203.72 € after 8 h hydrolysis. Alternatively, Alcalase plus Kojizyme, Novo Pro-D plus Flavourzyme, and Novozyme plus Flavourzyme can be used instead of Alcalase and Flavourzyme if the best one was limited by supplying and using in long-term. But Corolase LAP cannot be gained any profit, although this enzyme concentration was increased in the hydrolysis process. Given on their profits per cycle were summarized in Table 7.2. However, process economics can assist to decide which enzyme should be used.

Table 7.2 Profit of different combinations of enzyme hydrolyzed CGM for 10 tons of the dilution 8% protein (w/w) at 50°C, initial pH 9.0 for 8 h in 20 $m^3$ batch reactor.

| Combination of enzymes [E/S % (w/w)] | Profit*/cycle [€] |
|---|---|
| 2% Alcalase + 2.5% Flavourzyme | 5,200.65 |
| 2% NovoPro-D + 2.5% Flavourzyme | 2,224.67 |
| 2% Novozyme + 2.5% Flavourzyme | 1,930.33 |
| 2% Alcalase + 2.5% Kojizyme | 3,232.21 |
| 2% Alcalase + 2.5% Corolase LAP | -1,342.88 |
| 2% Alcalase + 5.0% Corolase LAP | -1,526.80 |

* Profit = Revenue - Expenses

## 7.2 Hydrolysis times

The harvest time was the number of hours per cycle that was taken at 2, 4, 6, 8, 10, 12, 16, 18, 22 and 24 h hydrolysis. As the amino acid products should be taken at 8 or 10 h because it can be operated max 2 cycles per day, depend on the capacity of plant. Thus, their profit were estimated about (5,200.61 × 2) = 10,401.21 € per day for cycle time of 8 h or (7,157.07 × 2) = 14,314.13 € per day for cycle times of 10 h (Figure 7.4). Although, the harvest time of 16-24 h gave higher profit per cycle, which were estimated mostly about 9,345.39 € per cycle but it can be operated only one cycle per day, thus their profit per day were lower than harvest time

at 8 or 10 h (Figure 7.3). Hence, harvest times at 6 and 4 h should be avoid due to obtain lower profit about of 7,676.03 and 1,732.77 € per day, respectively.

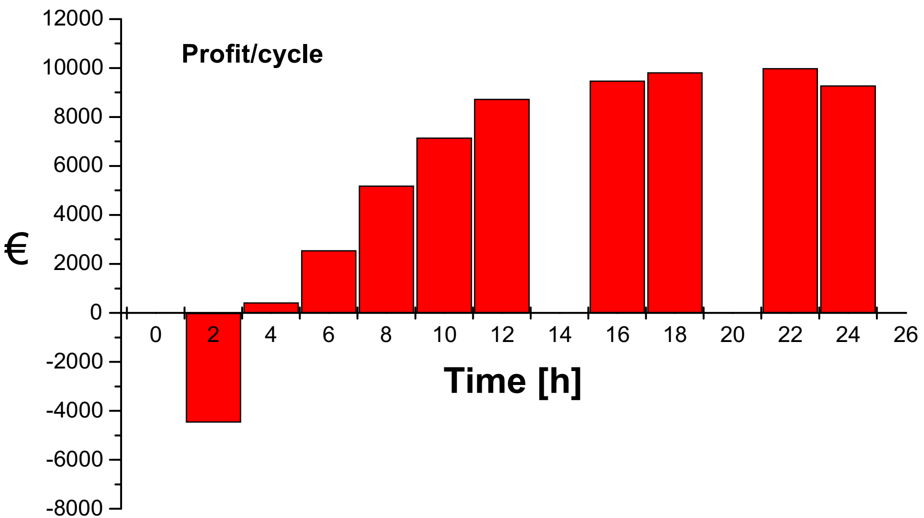



Figure 7.3 Profit per cycle of amino acid productions by enzymatic hydrolysis of corn gluten meal for 10 tons. Conditions; substrate conc. = 8% protein (w/w), enzyme conc. = 4.5% E/S [2% Alcalase + 2.5% Flavourzyme] at 50 °C, initial pH 9.0.

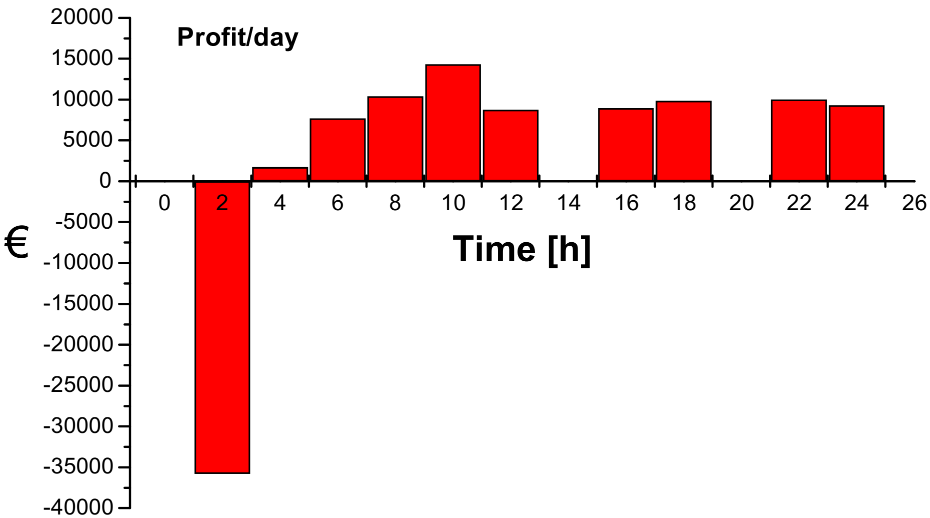



Figure 7.4 Profit per day of amino acid productions by enzymatic hydrolysis of corn gluten meal for 10 tons. Conditions; substrate conc. = 8% protein (w/w), enzyme conc. = 4.5% E/S [2% Alcalase + 2.5% Flavourzyme] at 50 °C, initial pH 9.0.

# 8 Conclusions

Renewable vegetable proteins such as corn gluten meal (CGM) and soybean meal (SBM) were hydrolyzed with commercially available enzymes and extremozymes. The hydrolysis process was carried out under mild condition, so that the nutritional quality of amino acid products was maintained for cosmetics, dietetics and pharmaceutical purposes.

Nitrogen content was analyzed by the Kjedahl method led to calculation crude protein (N × 6.25) content of CGM and SBM which were 63% and 50%, respectively. The amounts of crude protein were used to calculate the total hydrolysis ($h_{tot}$) of the degree of hydrolysis (DH).

Revered-phase HPLC were employed techniques for analysis of amino acids. Rapid and high sensitivity analysis of only primary amino acids were required. The degree of hydrolysis was also analyzed by o-phthaldialdehyde (OPA) and trinitro-benzene-sulfonic acid (TNBS) method, alternatively. These values were then compared using correlation analysis. The reaction time of OPA is 2 min, whereas the TNBS reaction needs an hour. In this case, OPA method can be used to monitor during the hydrolysis reaction.

Since the poteolytic activity of an enzyme affects to the different protein substrates. The activity of endopeptidase acted upon casein, while the activity of exopeptidase acted upon p-nitroaniline as substrate. Also the effect of pH and temperature on the enzyme action of more than 10 commercially available proteases were performed on vegetable proteins substrate such as CGM and SBM and were measured by HPLC method. However, intelligent screening of proteases does not follow a simple procedure, but requires a technical and economical evaluation of considerations.

As the degree of hydrolysis was related to enzyme kinetics after the effects of hydrolysis parameters such as enzyme dosages, enzyme combinations, substrate concentrations and reaction conditions were determined. The rate of hydrolysis was determined by measuring hydrolytic products as amino acids.

The process investment demanded reliable and steady performance from the catalysts. Thus, kinetic considerations were applied either a curve shape and the composition of the amino acids product or changing the hydrolysis conditions, for example, temperature or enzyme concentration. At high substrate concentrations, the reaction is of zero-order kinetic and the shape of reaction curve was steady state. Whereas, at low substrate concentrations, the reaction is of first-order kinetic and the shape of reaction curve increased proportionally with

substrate concentrations. The zero- and first-order kinetics suggested that the enzymatic hydrolysis was time dependent.

Heat treatment of the substrate prior to hydrolysis affected to the hydrolysis reaction, which was also derived from one by one versus zipper models. One by one type is slow initial steps but rapid decomposition of primary products. Zipper type kinetics show rapid initial steps but slower decomposition of primary and secondary products. The denaturation of protein was a major change from the original native structure without alteration of the amino acid sequence. The denaturation was considered accessible to enzyme attack led to improve hydrolysis rate. Thus, heat treatment prior to hydrolysis gave more percent of DH than without heat treatment about 5%.

The protein hydrolysis process was carried out in a 0.1 L reactor and was reproduced in a 4 L batch reactor, in case of laboratory experiments. However, the hydrolysis reaction was scale independent in well-stirred reactors. The combination of both enzymes between Alcalase (endopeptidase) and Flavourzyme (exopeptidase) was selected to use in the hydrolysis process because they appeared to possess the best combination of high activity and stability. The concentration of the combination was of 4.5% E/S (w/w), and was used in a two-step hydrolysis, which gave the DH values for CGM and SBM of 45% and 20% at the end of the hydrolytic process for 24 h. However, individual treatment with Flavourzyme reached 20% and 15% DH for CGM and SBM, respectively.

The velocities of hydrolysis were determined as amino acid concentrations [P] versus time. The velocity of progress curve was downward to steady state at the substrate saturation. However, the higher the substrate concentration, the more efficient will be the overall process economy. Thereby, the concentration of substrate should be at the saturation point. The substrate saturation of CGM and SBM were 8% and 10% protein, respectively. The hydrolysis process is recommended using the enzyme concentrations around 3.7-5% E/S (w/w) by using Alcalase (endopeptidase) about 0.5-2% and Flavourzyme (exopeptidase) about 1-2.5% E/S (w/w).

The effect of temperature on the reaction rate has several factors, for example, natural proteins, by-product inhibition and chemical lability. The initial velocities of hydrolysis curves were linear, but extent hydrolysis curves decline. Hence, at early time the rate of product formation corresponded to the uninhibited velocity of the enzymatic reaction. After 2 h of hydrolysis the velocity changed to the reflection of the ESP complex, which undergoes catalysis at a lower rate than the binary ES complex.

Alternatively, the enzymatic hydrolysis requires a stable high temperature protease. Extremozymes such as a recombinant carboxy-terminal protease from *Thermotoga maritima* and an isolated protease from *T. keratinophilus* were therefore tested. The characterization of both extremozyes were performed by hydrolyzing different protein substrates such as casein and CGM in order to express stability and altered specificity of extremozymes. It appears that the activities of both extremozymes were very low for hydrolyzing casein and CGM because there may be some substances in CGM which inhibited enzyme activity. Another reason, the hyperthermophiles can be expressed successfully in a mesophilic host such as *E. coli*, but difficulties might be encountered when the expressed enzymes requires specific cofactors or metal ions.

The process economics were calculated using 10 tons substrate per cycle. The effect of different endo- and exopeptidases on hydrolysis rate resulted in various amounts of amino acids. The costs of enzymes were also different. The enzymatic hydrolysis of CGM using the combination of Alcalase, an endopepidase and Flavourzyme, an exopeptidase obtained the highest profit about 5,200 € per cycle after 8 h of hydrolysis. Alternatively, Kojizyme, Novo Pro-D and Novozyme can be considered occasionally but we cannot gain any profit from Corolase LAP even using enzyme concentration increased to 5% in the process. The product should be taken at 8 or 10 h because of beneficial to operate 2 cycles per day. The result of harvesting product at 8 or 10 h per cycle gave the profit 10,401 or 14,314 € per day. These values were calculated only for hydrolyzing CGM.

# 9 Annex

## 9.1 Abbreviations

| | |
|---|---|
| AA | Amino acids |
| CGM | Corn gluten meal (a byproduct of the corn wet-milling process) |
| DH | Degree of hydrolysis. Defined as the percentage of peptide bonds cleaved during the proteolytic reaction. |
| E/S | Enzyme-substrate ratio (weight percent) |
| E | Enzyme (concentration) |
| $f_N$ | Kjeldahl conversion factor (6.25) |
| HPLC | High performance liquid chromatography |
| $h$ | mmoles analyzed for amino acid residues |
| $h_{tot}$ | Total mmoles of amino acid residues per g protein |
| N | Nitrogen (concentration) |
| OPA | *o*-phthaldialdehyde |
| P | Products |
| $r$ | A functional relationship |
| RSD | The relative standard deviation |
| S | Substrate concentration (weight percent N $\times f_N$) |
| SBM | Soybean meal (a by-product of the soybean crushing process) |
| SD | Standard deviation |
| TCA | Trichloroacetic acid |
| TNBS | Trinitrobenzenesulphonic acid |
| T | Temparature (°C) |
| $V_0$ | velocity or rate reactions |

## 9.2 Bradford assay

A rapid and reliable dye-based assay for determining protein content in a solution. Although there are relatively few interfering substances, the dye interacts more or less strongly with different purified proteins and thus is not strictly quantitative (Bollag and Edelstein, 1991).

### *A. Summary*

- Time required: 10 min.
- Advantages:
  1. Rapid (two minute development time)
  2. Sensitive, hence little protein must be sacrificed.
- Disadvantages:
  1. Some variability in response between different purified proteins.
  2. Proteins used for this assay are irreversibly denatured.

- Range of sensitivity: 25µg/ml – 200µg/ml protein solution; minimum volume of 0.1 ml permits measurement of as little as 2.5µg of protein.
- Theory: Bradford, 1979

***B. Equipment***

- Spectrophotometer
- Plastic cuvettes (polystyrene)
- Pastur pipets and pipet bulbs
- Pipets
- Small disposable test tubes or Eppendorf tubes
- Test tube rack
- Vortex mixer

***C. Reagents***

- Serva Blue G Dye
- 1 mg/ml bovine serum albumin (BSA)
- 95% ethanol
- 85% phosphoric acid

***D. Protocol***

- Solutions
  1. Bradford Stock Solution
     100 ml 95% ethanol
     200 ml 88% phosphoric acid
     350 mg Serva Blue G
     Stable indefinitely at room temperature.
  2. Bradford Working Buffer
     425 ml distilled water
     15 ml 95% ethanol
     30 ml 88% phosphoric acid
     30 ml Bradford Stock Solution

Filter through Whatman No.1 paper, store at room temperature in brown glass bottle. Usable for several weeks, but need to be refiltered.

- Assay
  1. Pipet protein solution (maximum 100µl) into tube (see example below for standard curve).
  2. Add experiment buffer to make a total volume of 100µl.
  3. Add 1 ml Bradford Working Buffer and vortex.
  4. Read $A_{595}$ after 2 minutes (Read and Northcote, 1981) but before 1 hour (Bearden, 1978).

## 9.3 HPLC analytical method

HPLC analysis of amino acids used to detect the rapid reaction of primary amines (does not react with secondary amino acids such as proline and hydroxyproline) with automatic pre-column OPA derivatization agent to from a highly fluorescent product (Pfeifer et al., 1983).

***Instruments and Method***

Pump: gradient pump; CP4100, Techlab
Degasser: ERC-3322, Techlab
Injector: Triathlon autosampler, Spark
Detector: RF 10AXL fluorescence detector, Shimadzu
Column: Resolve$^{TM}$ 5 µm $C_{18}$ , 4 × 150 mm, Reversed phase Waters

Mobile phase A: 0.05 M sodium acetate, pH 7.0 : Methanol : Tetrahydrofuran (1000:21:21)
Mobile phase B: Methanol : distilled water (27:23)
Flow rate : 1.0 ml/min
Detection : Excitation, 330 nm; Emission, 420 nm
Derivatization: OPA/2-mercaptoethanol

***Reagents***

1. Acetate buffer, 0.05 M , pH 7.

   Sodium acetate, Mw: 82.03 ; 13.6g and 12 g $NaH_2PO_4$ dehydrate dissolved with distilled water to 2 L, added 5 ml 10 M NaOH, adjust pH to 7.0 with NaOH.
2. Mobile phase A

   Acetate buffer (1), 2000 ml : 42 ml Methanol : 42 ml Tetrahydrofuran
3. Mobile phase B

   920 ml Methanol : 1,080 ml distilled water
4. Borate buffer, 0.4 mol/l, pH 9.5

   Boric acid, $H_3BO_3$ 2.47g dissolved with distilled water to 90 ml, adjust pH to 9.5 with NaOH and adjust to 100 ml.
5. Derivatization reagent, OPA 40 mmol/l

   *o*-phthaldialdehyde, $C_8H_6O_2$ 270 mg in 5 ml Ethanol, 200 µl Mercaptoethanol added with 50 ml 0.4 M Borate buffer pH 9.5

***Calibrator***

Amino acids calibrator, 18.75 µmol/l in distilled water

Sigma, AA-S-18; the components are alanin, ammonium chloride, arginin, aspatic acid, cystine, glutamic acid, glycine, histidine, isoleucine, lysine, methionine, phenylalanine,

proline, serine, threonine, tyrosine and valine. If additional amino acids are to be analyzed, they can be added to the calibration solution.

***Analysis***

Let the pumps run with eluents for at least 30 minutes to equilibrate the column. Inject 10 µl of distilled water to check that there are no extra peaks.

1. Pipette 10 µl of samples to be analyzed (calibrators and unknowns) into sample vials and place in autosampler.
2. Add derivatization reagent (5) to vial in reagent position in autosampler.
3. Program autosampler to add 10 µl derivertization reagent to sample to be injected. Let react for 60 seconds before injection.

***o-phthalaldehyde***

Primary amino acids will react with *o*-phthalaldehyde in the presence of the strongly reducing 2-mercaptoethanol (pH 9-10) to yield a fluorescent product (emission maximum, 455 nm; excitation maximum, 340 nm). Peptides are less reactive than ∝-amino acids and secondary amines do not react at all. As a result, proline and hydroxyproline must first be treated with a suitable oxidizing agent such as chloramine T (sodium *N*-chloro-*p*-toluene-sulphonamide) or sodium hypochlorite, to convert them into compounds which will react. Similarly cystine and cysteine should also be first oxidized to cysteic acid. The fluorescent yield of individual amino acids varies and fluorescence values must be determined for accurate quantitative work in the same manner as the colour values for ninhydrin.

The aqueous reagent is stable at room temperature and the reaction proceeds quickly without requiring heat. The method is approximately ten times more sensitive than the ninhydrin method and is particularly useful when the quantity of many amino acids is being carried out using amino acid analysers or HPLC.

The fluorescence detection of amino acids used the *o*-phthaladehye/2-mercaptoethanol (OPA/MCE). The reaction OPA/MCE adduct, which then interacted with the primary amine on amino acid to form a fluorescent 1-alkyl thio-2-alkyl-substituted isoindole. The reaction was rapid and quantitative but isoindoles produced were unstable due to a sulfur-oxygen rearrangement to produce a non-fluorescent 2,3-dihydro-1H-isoindole-1-one.

$R - NH_2$ +

Primary amine

Fluorescamine
(non-fluorescent)

Fluorescent product

Figure 9.1 The reaction of fluorescamine with a primary amino acid group. The reaction of an amino acid containing a primary amino group with a solution of fluorescamine in acetone at pH 9.0 results in the conversion of the non-fluorescent fluorescamine to a fluorescent product.

## 9.4 Determination of crude protein (Kjeldahl method)

***The determination of Kjeldahl N*** The procedures are adapted from those of the Association of Official Analytical Chemists (AOAC, 1975) and are described for both macro and semi-micro scale. The macro Kjeldahl procedure is for samples difficult to homogenize, and the sample size is between 1-3 g; the semi-micro determination is designed for small quantities (< 300 mg) of homogeneous material. Numerous modifications of the original procedure have been proposed (Munro and Fleck, 1969).

The sample should be well mixed with no coarse particles or in homogenous (Novo Industri A/S, 1978c). An amount of sample corresponding to 2-25 mg N is added into a tare digestion tube. A digestion tablet (1.5 g $K_2SO_4$ with 0.0075 g Se) is added, and 3 ml conc. $H_2SO_4$ is dispensed into the tube (if the sample contains above 50% carbohydrate, the amount of $H_2SO_4$ should be 6 ml). 1.5 ml 35% $H_2O_2$ may be added (cautiously).

Digestion is carried out at 420°C, usually for half an hour. After cooling, the tube is placed in the distillation unit, excess (25 ml) 35% NaOH is added and the liberated $NH_3$ is steam distilled for two minutes (distillation volume 95-100 ml) into 25 ml receiver solution containing 1% boric acid with bromocresol/methyl red indicator. The receiver solution is titrated with 0.1 N HCl.

***Calculation of crude protein content in the dry matter for an example***

| | Variable | Calculation | Results/Examples | |
|---|---|---|---|---|
| Samples: | | | 1 | 2 |
| a) | Fresh sample weight (g) | | 1.0000 | 1.0000 |
| b) | Dry sample weight (g) | | 0.9049 | 0.9049 |
| c) | Normalitiy of HCl = 0.1 N, 0.25 N | | 0.1 N | |
| | 1 ml of 0.1 N HCl corresponds to 1.4 mg Nitrogen. | | | |
| | 1 ml of 0.25 N HCl corresponds to 3.5 mg Nitrogen | | | |
| d) | Correction factor for HCl 20 ml of a 0.1 N standard solution was distilled and titrated with 19.8 ml HCl of ~ 0.1 N instead of 20 ml 0.1 N HCl | | | |
| | Correction factor therefore = 20/19.8 = 1.01 | | | |
| e) | Titration HCl in ml | | 11.2 | 11.1 |
| f) | N % | = (e × d × c)/(b × 10) | 1.75 | 1.73 |
| g) | Crude protein % | = f × 6.25 | 10.94 | 10.81 |
| | | **Average** | **10.88** | |

## 9.5 Correlation analysis

Whereas the regression function indicates the type of the relationship, correlation analysis investigates the degree of dependence between two or more random variables (Chapman and Shorter, 1978; Massart et al., 1978). The most important measure of dependence is the correlation coefficient *p*. An assume values between +1 and –1. If $r = 1$ or –1, a functional relationship exists between *x* and *y*. If $r = 0$, no relationship exists between *x* and *y* (Bergmeryer, 1986).

***Calculation***

Let a random sample of a two-dimension *xy*-population be given. The random sample consists of *n* pairs of values $(x_1y_1)(x_2y_2),\ldots,(x_ny_n)$. Then $\bar{x} = (x_1+x_2+\ldots+x_n)/n$ is the mean of the *x* values in the random sample. The variance of these figures is $S_x^2$ resp. $S_y^2$.

The product of the two deviations of the observed values $(x, y)$ from the corresponding mean $(\bar{x}, \bar{y})$ is taken as a measure of the covariances ($S_{xy}$) of the random sample. This is calculated as

$$S_{xy} = \frac{1}{n-1}\sum_{i-1}^{n}(x_i - \bar{x})(y_i - \bar{y}) \qquad \text{The quotient} \quad r = \frac{S_{xy}}{S_x Y_y}$$

is called the correlation coefficient of the random sample. The following formula is more suitable for calculation:

$$r = \frac{\sum x_i . y_i - \frac{1}{n} \sum x_i \sum y_i}{\sqrt{\left[ \left( \sum x_i^2 - \frac{1}{n} \left( \sum x_i \right)^2 \right) \left( \sum y_i^2 - \frac{1}{n} \left( \sum y_i \right)^2 \right) \right]}}$$

Obviously, the concept of correlation is not restricted to linear relationships but can be generally used. A wide field of application of linear regressions is the graphical comparison of different methods of clinical chemistry where it is very often used besides other statistical tools which are designed for the correct statistical comparison.

Two of these procedures are especially for comparing two chemical methods.

- The standardized principle component method (Feldmann, et al., 1981),
- a newly developed statistical method which is distribution-free (Passing and Bablok, 1983).

As the comparison of different groups, the distribution-free regression and correlation analyses which use the principle of the ranks of the observations. They are known as *Kendall's* τ and *Spearman's* rank correlation coefficient, respectively (Cornbleet and Shea, 1978).

***Precision***

The agreement between values from replicate measurements is defined as *precision*. The magnitude of the precision is expressed by the *imprecision*, characterised by the standard deviation (SD):

$$\text{SD} = \sqrt{\frac{\sum \left( x_i - \bar{x} \right)^2}{n-1}}$$

## 9.6 Endopeptidase assay

A protease is allowed to hydrolyze casein for 20 minutes at 35 °C. Undigested protein is precipitated with trichloroacetic acid and the quantity of digested product is determined by spectrophotometer at 280 nm (Bergmeyer, 1974).

***Reagents***

1. Casein (Merck, Darmstadt)

2. $KH_2PO_4$
3. $Na_2HPO_4.2H_2O$
4. Trichloroacetic acid (TCA), 5% (w/v)
5. HCL, 1 mN

***Solution***

I. Phosphate buffer (0.1 M, pH 7.6):

0.157 g $KH_2PO_4$ and 1.575 g $Na_2HPO_4.2H_2O$ dissolved in 90 ml distilled deionized water, adjusted pH to 7.6 before add water to 100 ml.

II Substrate solution:

1 g Casein dissolved completely with 100 ml phosphate buffer (solution I) about 15 min on hot plate. Adjust volume to 100 ml with water. The casein solution, pH values must be kept above 6.0 to prevent precipitation of the protein.

Keep cool at 0-4 °C to protect microorganism growth. Keep on the buffer solution for 1 year and casein solution minimum 1 week.

***Procedure***

Spectrophotometer to read absorbance at 280 nm; quartz cuvette, stratum thick: 1 cm. Incubation temperature: 35 °C; incubation volume: 2 ml; measurement against water. Preheat water bath to 35 °C (approx. 5 min). The solution contains 1-25 µg proteases (Bergmeyer, 1974)

| In 12 ml centrifuge tub (in 35 °C water bath) | Sample | Blank | Concentration in test |
|---|---|---|---|
| Sample (1-25µg proteases in 1 mN HCl)+phosphate buffer (I) | 1 ml | -- | 50-100mM phosphate, |
| Preheat substrate solution (II) | 1 ml | 1 ml | 5 mg casein/ml |
| mixed, stopwatch for 20 min incubation | | | |
| 5% trichloroacetic acid (TCA) | 3 ml | 3 ml | 3% |
| Sample + phosphate buffer | -- | 1 ml | |
| well mixed, leave minimum 30 min at room temperature. Centrifuge at 13,000×g for 10 min. Read absorbance at 280 nm. | | | |

***Calculation***

The activity of enyme ($TU^{cas}$)

$U^{cas}$ /µg trypsin = $\frac{0.25}{2.1\times 20}$ = $6\times10^{-3}$, that is 1 mg trypsin contains 6 $TU^{cas}$

0.25 = Δ absorbance 280/20 min

2.10 = µg trypsin/ml

20 = convert $A_{280}$/20 min to $A_{280}$/min

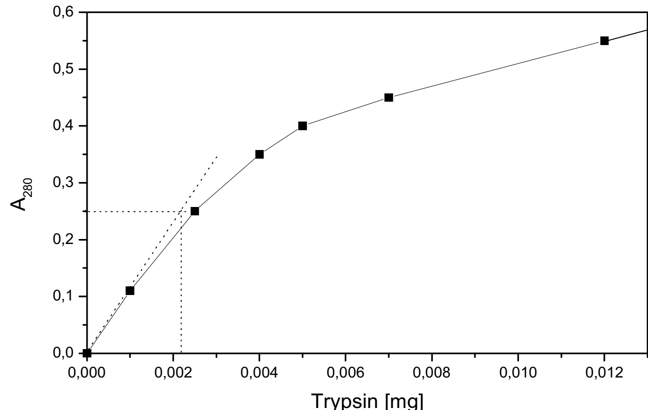



Figure 9.2 Standard curve for trypsin is allowed to hydrolyze casein.

The volume of incubation: 2 ml; casein concentration: 0.5%; 20 min incubation at 35°C; The volume after added TCA: 5 ml; the final concentration: 3%.

***Abscissa***: µg trypsin in 1 ml incubation volume

***Ordinate***: read absorbance at 280 nm from trypsin is allowed to hydrolyze casein in 20 min.(1 cm stratum thick; 5 ml total volume).

***Superior scale***: $10^{-3}$ $TU^{cas}$ in 1 ml incubation preparation.

## 9.7 Exopeptidase assay

Leucine aminopeptidase, EC 3.4.11 LAPU (leucine arylamidase), hydrolyzes L-leucine-p-nitroanilie to form p-nitroaniline which is measured using a photometer at 405 nm. 1 leucine aminopeptidase unit (LAPU) is the amount of enzyme which hydrolyzes 1 mmol substrate per minute under the given standard conditions (Novo/EB-SM-0296.02/01).

***Reagent/substrates***

1. Hydrochloric acid (HCl), 2M
2. Tris buffer 0.1M pH 8.0; 100 ml 1 M Tris buffer with 32 ml 2 M HCl added distilled deionized water up to 1 L, pH adjusted to 8.0 using NaOH or HCl as appropriate.
3. L-leucine-p-nitroanilide 26 mM (LNA-R); dissolved with 96% ethanol (prepared immediately before use. Protect from light (tin foil).
4. 6% ethanol

***Samples and standards***

The sample is diluted to 0.05 LAPU/ml (OD 0.1-0.4) with Milli-Q water. The samples must be clear (centrifuged if necessary) and analysed immediately after dilution. The analysis is performed relative to a known leucine aminopeptidase serving as a control for the level of the run (level control). The level control is a representative aminopeptidase production batch.

***Procedure***

The reagents are prepared. The sample and controls are diluted to approx. 0.05 LAPU/ml in Eppendorf (vol. 2 ml) and analysed as shown in the table below (Standard method, Novo Nordisk/EB-SM-0298.02/01).

| Tris buffer 0.1 M pH 8.0 | 1 ml |
|---|---|
| Sample | 66.5 µl |
| Heated to 40 °C for 15 min | |
| LNA-R (stopwatch) | 66.5 µl |
| Mixed, incubated at 40 °C for 10 min | |

***Photometer*** The sample are analysed using a spectrophotometer after 1 minute and again after 11 minutes (incubation time 10 minutes) at $\lambda$ = 405 nm. The cuvette is rinsed with 6% ethanol between each sample.

*All waste products from the LAPU analysis are collected in a waste container. The container is marked “0.5% L-leucine-p-nitroanilid” and “extremely poisoning”.

***Calculation*** $\Delta OD = OD_{sample(11\ min)} - OD_{sample\ (1\ min)}$

$$\text{LAPU/ml} = \frac{\Delta OD \times 1.133}{10 \times 9.62 \times 0.0665} \times D = \Delta OD \times 0.177 \times D$$

| | | |
|---|---|---|
| 1.133 ml | = | total volume |
| 0.0665 ml | = | volume of enzyme sample |
| 10 min | = | incubation time in minutes |
| 9.62 | = | micro molar extinction coefficient, mmol/ml |
| D | = | dilution factor |

### 9.8 Calculation of DH%

The degree of hydrolysis (% DH), defined as the mole % of amino acids (i.e., residues per 100 residues), is a key parameter which characterizes a protein by hydrolysate:

$$\% \text{ DH} = \frac{h}{h_{\text{tot}}} \times 100\,\%$$

$$= \frac{\text{mmoles analyzed for amino acid residues}}{\text{Total mmoles of amino acid residues/g protein}} \times 100\%$$

$h$ = mg analyzed for each amino acid divide by the mg of each residue molecular weight

$h_{\text{tot}}$ = 1000 mg of amino acid residue divide by the average mg of residues molecular weight (121 mg).

For example:

$$h = \sum_{i=m}^{n} \frac{\text{mgAA}_\text{i} \,/\, \text{g protein}}{\text{MW}_{\text{AA}_\text{i}} - \text{MW}_{\text{H}_2\text{O}}} \;;\; \text{m, n} \in \{1,2,3,...,18\},\ \text{m} < \text{n}$$

$$= \frac{200 \text{ mg Alanine}}{[89 - 18]} + \ldots + \text{n}$$

$$h_{\text{tot}} = \frac{1000 \text{ mg}}{\text{average}\,[\text{MW}_{\text{AA}} - \text{MW}_{\text{H}_2\text{O}}]}, \qquad = \frac{1000}{[139 - 18]} = 8.26$$

The protein in question does not have any prolines and cysteines. Otherwise, the sum of the amino acids is greater than 1000 mg/g protein because the amino acids include the water that was lost in forming the peptide bond. Thus, the amino acid residue is the form of the amino acid as exists in the protein, that is, the amino acid minus water or 18 less than the molecular weight. The molecular weight must be known or an assumed value used in order to calculates per molecule.

$$\text{mmol} = \frac{\text{wt}_{\text{mg}}}{\text{MW}}$$

### 9.9 TNBS assay

An accurate, reproducible and generally applicable procedure for determining the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid has been developed. The protein hydrolysate is dissolved/dispersed in hot 1% sodium dodecyl sulfate to a concentration of 0.25-2.5 × $10^{-3}$ amino equivalents/L. A sample solution (0.250 ml) is mixed with 2.00 ml of 0.2125 M sodium phosphate buffer (pH 8.2) and 2.00 ml of 0.10%

trinitrobenzenesulfonic acid, followed by incubation in the dark for 60 min at 50°C. The reaction is quenched by adding 4.00 ml of 0.100 N HCl, and the absorbance is read at 340 nm. A 1.500 mM L-leucine solution is used as the standard. Transformation of the measured leucine amino equivalents to degree of hydrolysis is carried out by means of a standard curve for each particular protein substrate (Adler-Nissen, 1979).

***Reagents***;

1. Trinitrobenzenesulfonic acid dihydrate (analytical grad, Fluka 29824)
2. $NaDodSO_4$ (SDS) (Serva 20760)
3. $NaH_2PO_4$ (Merck 105108)
4. $Na_2HPO_4$ (Merck 105101)
5. L-leucine (Riedel-de Haen 41283)

***TNBS solution***; TNBS is dissolved in deionized water in a volumetric flask (100 or 150 ml) covered with aluminium foil; the solution must be prepared immediately before use.

***Phosphate buffer***; 0.2125 M $NaH_2PO_4$ is added to 0.2125 M $Na_2HPO_4$ until pH is 8.20 ± 0.02 (the proportion of volumes is approximately 43:1000)

***Standard solution***; 0.1 M L-leucine standard in 1% $NaDodSO_4$ (SDS is dissolved in phosphate buffer, to keep cooling below room temperature may came turbidity).
The sample should be dispersed in sodium dodecyl sulfate ($NaDodSO_4$) alone.

## 9.10 OPA assay

Another alteration to determine the degree of hydrolysis (DH) is the method based on the reaction of primary amino groups with o-phthaldialdehyde (OPA). Serine was the standard used, since in reactions serine shows a response very close to the average response of amino acids at 340 nm in a spectrophotometer (Nielsen, 2001).

***Reagents***;

1. di-Na-tetraborate decahydrate (Merck 2725721, Darmstadt)
2. Na-dodecyl-sulfate (SDS) (Serva 20760)
3. o-phthaldialdehyde 97% (OPA) (Sigma P 1378, Deisenhofen)
4. dithiothreitol 99% (DTT) (Merck 11474, Darmstadt)
5. serine (Sigma S 4250, Deisenhofen)

***OPA solution***;

- 7.620 g di-Na-tetraborate decahydrate and 200 mg Na-dodecyl-sulfate (SDS) were dissolve in 150 ml deionized water. The reagents were completely dissolved before continuing.

- 160 mg *o*-phthaldialdehyde 97% (OPA) was dissolved in 4 ml ethanol.
- The OPA solution was then transferred quantitative to the above mentioned solution by rinsing with deionized water.
- 176 mg dithiothreitol 99% (DTT) was added to the solution by rinsing with deionized water.
- The solution was made up to 200 ml with deionized water.

***Standdard solution***; the serine standard was prepared as follows: 50 mg serine (Art.7769 Merck, Darmstadt, Germany) was diluted in 500 ml deionized water (0.9516 meqv/l)

The sample solution was prepared as follows: X g sample was dissolved in 100 ml deionized water. X is 0.1 to 1.0 g sample containing 8% to 80% protein. The DH of the sample also influences the amount required.

***Procedure***; spectrophotometer was performed at 340 nm using deionized water as the control. The OPA reagent (3 ml) was added to all test tubes with 400µl sample solution. As absorbance changes somewhat with time, it is importance the sample stand for exactly the same time (2 min) before measuring. The assay was carried out at room temperature.

## 9.11 Product data of enzyme supplied by the companies

| Enzyme | EC number | Company | Enzyme activity | pH opt. | Temp. opt. |
|---|---|---|---|---|---|
| Alcalase 2,5 L DX | 3.4.21.62 | Novo | 2.5 AU/g | 6.5-9 | 50-70°C |
| Alcalase 2,4 L | 3.4.21.62 | Novo | 2.4 AU/g | 6.5-9 | 50-70°C |
| Savinase 16,0L EX | 3.4.21.62 | Novo | 16 KNPU/g | 8.0-10.0 | 50°C |
| Esperase 8,0L | 3.4.21.62 | Novo | 8 KNPU/g | 7.5-10.5 | 55-60°C |
| Novo Pro-D | 3.4.21.62 | Novo | 16 KNPU/g | ca.6.5 | 55-65°C |
| Novozyme FM 2,0L | 3.4.21.62 | Novo | 2.0 AU/g | 6.5-12.0 | 50-65°C |
| Neutrase 0,8L | 3.4.21.62 | Novo | 0.8 AU/g | 5.5-7.5 | 45-55°C |
| AMI 00.01 | 3.4.24.-- | DSM | 70 PCU/g | 5.0-8.0 | 55°C |
| AMI 00.02 | 3.4.22.-- | DSM | 5100 NFPU/mg | 5.0-7.0 | 60-70°C |
| AMI 00.03 | 3.4.21.-- | DSM | 560 DU/g | 7.5-9.5 | 60-65°C |
| Corolase S50 | 3.4.21.62 | Röhm | > 3785 UHb/g | 5.0-8.0 | 55-65°C |
| Corolase PN-L | 3.4.21.63 | Röhm | 250 UHb/g | 5.0-7.0 | 40-50°C |
| Corolase LAP | 3.4.11.1 | Röhm | 350 LAPU/g | 7.0-9.0 | 60-70°C |
| Kojizyme 500MG | 3.4.11.1 | Novo | 500 LAPU/g | 5.5-6.5 | 30°C |
| Flavozyme 1000L | 3.4.11.1 | Novo | 1000 LAPU/g | 5.0-7.0 | 40-60°C |

***The declared activity***; Anson Units (AU)

Leucine Aminopeptidase Units (LAPU)

Kilo Novo Protease Units (KNPU)

***Company address***; Novo Nordisk, Denmark/ Gamma Chemie GmbH, München

Röhm Enzyme GmbH, Damstadt

DSM Food Specialties, France

## 9.12 Raw data of amino acids composition

Table 9.1 Amino acid composition of corn gluten meal 8% protein (w/w) was hydrolyed with 2% Alcalase 2,4L plus 2.5% Flavourzyme 1000L E/S% (w/w) at 50°C, pH 9.0 in 4 L batch reactor (non-pretreatment).

| Amino acid [kg/t] | 2h | 4h | 6h | 8h | 10h | 12h | 16h | 18h | 22h | 24h |
|---|---|---|---|---|---|---|---|---|---|---|
| Asp | 0.454 | 0.468 | 1.5625 | 1.7455 | 1.289 | 0.5514 | 1.1302 | 1.5203 | 1.281 | 1.1714 |
| Glu | 0.8993 | 1.5106 | 2.0051 | 3.3131 | 3.8566 | 3.439 | 4.0178 | 4.4079 | 4.1686 | 4.059 |
| Asn | 1.8536 | 2.634 | 9.1999 | 9.5079 | 10.0514 | 9.6647 | 10.2435 | 10.6336 | 10.3943 | 10.2847 |
| Ser | 2.9671 | 3.6399 | 10.4797 | 10.7877 | 10.3312 | 10.452 | 11.0308 | 10.4209 | 10.1816 | 10.072 |
| Gln | 17.3719 | 29.6307 | 34.2927 | 35.6007 | 36.1442 | 38.246 | 38.8248 | 39.2149 | 40.9756 | 40.866 |
| His | 1.9547 | 2.0593 | 2.2999 | 2.6079 | 3.1514 | 3.719 | 4.2978 | 3.6879 | 3.4486 | 3.339 |
| Gly | 3.7138 | 3.8743 | 5.5442 | 5.8522 | 6.3957 | 6.5581 | 7.1369 | 7.527 | 7.2877 | 7.1781 |
| Thr | 2.503 | 2.9933 | 8.6055 | 9.9135 | 10.457 | 11.1117 | 11.6905 | 12.0806 | 12.8413 | 11.7317 |
| Arg | 2.4741 | 2.692 | 9.9549 | 10.2629 | 10.8064 | 11.4607 | 12.0395 | 11.4296 | 11.1903 | 11.0807 |
| Ala | 3.1918 | 4.0247 | 14.5972 | 16.9052 | 17.4487 | 17.4454 | 18.0242 | 18.4143 | 18.175 | 18.0654 |
| Tyr | **5.5648** | **7.2815** | **11.773** | **14.081** | **15.6245** | **16.6463** | **17.2251** | **17.6152** | **17.3759** | **17.2663** |
| Met | 1.9688 | 3.8379 | 7.6879 | 7.9959 | 8.5394 | 8.0806 | 8.6594 | 8.0495 | 8.8102 | 8.7006 |
| Val | 2.7439 | 6.4248 | 10.7939 | 15.1019 | 15.6454 | 16.43 | 17.0088 | 17.3989 | 16.1596 | 16.05 |
| Trp | 1.0781 | 0.5565 | 0.1939 | 1.1141 | 1.6576 | 1.2441 | 1.8229 | 1.9213 | 1.9737 | 1.8641 |
| Phe | **8.0888** | **11.7343** | **15.8031** | **17.1111** | **18.6546** | **19.6457** | **21.2245** | **21.6146** | **21.3753** | **20.2657** |
| **Ile** | **4.9435** | **6.3043** | **9.5804** | **10.8884** | **11.4319** | **12.2246** | **12.9034** | **13.1935** | **13.9542** | **13.8446** |
| **Leu** | **20.2008** | **33.4162** | **40.649** | **45.957** | **49.5005** | **53.7093** | **54.5881** | **55.6782** | **57.4389** | **57.3293** |
| Lys | 3.4639 | 7.8505 | 7.2598 | 7.5678 | 5.1113 | 8.3277 | 8.3065 | 8.2966 | 7.0573 | 8.9477 |
| Total | **85.4359** | **130.9328** | **202.2826** | **226.3138** | **236.0968** | **250.9563** | **260.1742** | **263.1044** | **264.0891** | **262.1163** |

Table 9.2 Amino acid composition of corn gluten meal 8% protein (w/w) was hydrolyed with 2% Alcalase 2,4L plus 2.5% Flavourzyme 1000L E/S% (w/w) at 50°C, pH 9.0 in 4 L batch reactor (pretreatment by heating at 80°C for 2 h prior to hydrolysis).

| Amino acid [kg/t] | 2h | 4h | 6h | 8h | 10h | 12h | 16h | 18h | 22h | 24h |
|---|---|---|---|---|---|---|---|---|---|---|
| Asp | 0.6228 | 0.7878 | 1.0385 | 1.0419 | 0.9762 | 0.9221 | 1.2554 | 1.6784 | 1.289 | 1.2679 |
| Glu | 1.0032 | 1.3841 | 2.1182 | 2.3576 | 2.6591 | 3.1127 | 3.446 | 3.869 | 5.462 | 5.4359 |
| Asn | 3.5377 | 5.3895 | 8.2937 | 10.6152 | 11.0918 | 12.3113 | 12.6446 | 13.0676 | 14.5566 | 13.6905 |
| Ser | 4.7853 | 6.8443 | 10.7557 | 10.1451 | 10.5251 | 10.6501 | 10.9834 | 11.4064 | 6.2411 | 5.8837 |
| Gln | 18.9104 | 28.8535 | 44.3429 | 46.1317 | 48.3702 | 53.2295 | 53.5628 | 53.9858 | 55.5595 | 54.0591 |
| His | 2.1421 | 3.1782 | 5.0427 | 4.9002 | 5.5601 | 5.5188 | 5.8521 | 6.2751 | 6.6574 | 6.6153 |
| Gly | 1.9972 | 2.6637 | 4.055 | 5.5884 | 6.5302 | 6.134 | 6.4673 | 6.8903 | 6.6607 | 7.2886 |
| Thr | 3.3525 | 5.7004 | 8.6924 | 9.4137 | 10.3614 | 11.5456 | 11.8789 | 12.3019 | 14.0243 | 13.8122 |
| Arg | 3.9545 | 5.74 | 8.2478 | 8.512 | 7.709 | 4.9028 | 5.2361 | 4.6591 | -- | -- |
| Ala | 5.8845 | 9.0129 | 13.9503 | 14.9267 | 16.3367 | 18.7777 | 18.111 | 18.534 | 20.8033 | 19.9179 |
| **Tyr** | **4.5708** | **8.518** | **11.0002** | **13.2427** | **14.1615** | **17.597** | **17.9303** | **18.3533** | **18.9582** | **18.003** |
| Met | 3.1885 | 4.6324 | 6.8356 | 6.7696 | 6.9036 | 7.4752 | 7.8085 | 8.2315 | 8.5791 | 7.9859 |
| Val | 4.5614 | 7.1748 | 10.0673 | 12.029 | 12.5116 | 13.6007 | 13.934 | 14.357 | 16.4825 | 15.6361 |
| Trp | 1.1717 | 1.6971 | 2.1784 | 2.8508 | 2.8964 | 2.9912 | 3.3245 | 3.7475 | 3.1599 | 3.1501 |
| **Phe** | **8.394** | **12.432** | **16.754** | **18.9086** | **19.3883** | **21.009** | **22.3423** | **22.7653** | **23.9873** | **23.0302** |
| **Ile** | **4.754** | **7.2926** | **9.7278** | **11.4245** | **11.6983** | **12.5884** | **12.9217** | **13.3447** | **14.9638** | **14.2406** |
| **Leu** | **20.2269** | **32.262** | **43.9228** | **53.8327** | **55.3208** | **60.1654** | **60.4987** | **60.9217** | **61.4559** | **58.8809** |
| Lys | -- | -- | -- | 7.8173 | 7.5662 | 7.9317 | 8.265 | 3.188 | 8.1554 | 7.1063 |
| **Total** | **93.0572** | **143.5633** | **207.0232** | **240.5076** | **250.5665** | **270.4632** | **275.4631** | **277.577** | **286.996** | **276.0042** |

Table 9.3 Raw data of enzymatic hydrolysis of soy protein. Conditions; substrate conc.: 8% protein (w/w), enzyme conc.: 2% Alcalase2,4 L FG and 2.5% Flavourzyme 1000L (w/w), 50°C, initial pH 8.5 in 4 L batch reactor (non-pretreatment).

| Amino acids [Kg/t] | 4h | 6h | 8h | 14h | 24h |
|---|---|---|---|---|---|
| Asp | 1.0633 | 1.1433 | 1.6677 | 2.0179 | 2.2212 |
| Glu | 3.6792 | 4.2894 | 5.2929 | 5.6431 | 5.8464 |
| Asn | 3.7623 | 4.4802 | 5.0743 | 5.4245 | 5.6278 |
| *Ser* | 3.3192 | 3.7226 | 4.2546 | 4.6048 | 4.8081 |
| *Gly* | 2.2018 | 2.5622 | 3.5771 | 3.9273 | 4.1306 |
| *His* | 1.6978 | 2.0199 | 2.6342 | 2.9844 | 3.1877 |
| Gly | 1.3041 | 1.3203 | 1.7358 | 2.086 | 2.2893 |
| Thr | 2.4154 | 2.9549 | 4.2392 | 4.5894 | 4.7927 |
| Arg | 5.6978 | 6.8888 | 0.6175 | 0.9677 | 1.171 |
| Ala | 2.3273 | 2.6589 | 3.3013 | 3.6515 | 3.8548 |
| **Tyr** | **4.1574** | **4.6591** | **5.1383** | **5.4885** | **5.6918** |
| Met | 1.6943 | 1.9599 | 2.6035 | 2.9537 | 3.157 |
| Val | 3.8067 | 4.7199 | 5.5153 | 5.8655 | 6.0688 |
| Trp | 1.8133 | 2.0199 | 2.4612 | 2.8114 | 3.0147 |
| **Phe** | **5.9762** | **7.427** | **8.7811** | **9.1313** | **9.3346** |
| **Ile** | **3.0866** | **3.9858** | **5.2081** | **5.5583** | **5.7616** |
| **Leu** | **8.6042** | **10.8713** | **12.2366** | **12.5868** | **12.7901** |
| Lys | 7.0527 | 6.6104 | 6.0844 | 6.4346 | 6.6379 |
| **Total** | **63.6595** | **74.2939** | **80.4228** | **86.7267** | **90.3861** |

Table 9.4 640% (w/w) extremozyme from *T. keratinophilus* hydrolyzed 8% protein CGM.

| Amino acid [kg/t] | 2h | 4h | 6h | 8h | 10h | 12h | 16h | 18h | 22h | 24h |
|---|---|---|---|---|---|---|---|---|---|---|
| Asp | 0.1313 | 0.1361 | 0.1381 | 0.1209 | 0.1387 | 0.2427 | 0.3287 | 0.4063 | 0.4683 | 0.5184 |
| Glu | 0.2819 | 0.2867 | 0.2682 | 0.2801 | 0.2991 | 0.4031 | 0.4891 | 0.5667 | 0.6287 | 0.6788 |
| Asn | 0.1697 | 0.2308 | 0.2699 | 0.3167 | 0.3957 | 0.4997 | 0.5857 | 0.6633 | 0.7253 | 0.7754 |
| Ser | 0.2319 | 0.2821 | 0.3416 | 0.3123 | 0.3693 | 0.4733 | 0.5593 | 0.6369 | 0.6989 | 0.749 |
| Gln | 0.0777 | 0.1467 | 0.1643 | 0.1862 | 0.2068 | 0.3108 | 0.3968 | 0.4744 | 0.5364 | 0.5865 |
| His | 0.1441 | 0.1495 | 0.1574 | 0.1538 | 0.1955 | 0.2995 | 0.3855 | 0.4631 | 0.5251 | 0.5752 |
| Gly | 0.1269 | 0.1341 | 0.1508 | 0.1393 | 0.1484 | 0.2524 | 0.3384 | 0.416 | 0.478 | 0.5281 |
| Thr | 0.1395 | 0.1699 | 0.1936 | 0.2056 | 0.2302 | 0.3342 | 0.4202 | 0.4978 | 0.5598 | 0.6099 |
| Arg | 0.0862 | 0.1189 | 0.1388 | 0.1673 | 0.199 | 0.303 | 0.389 | 0.4666 | 0.5286 | 0.5787 |
| Ala | 0.53 | 0.625 | 0.7064 | 0.7944 | 0.9165 | 1.0205 | 1.1065 | 1.1841 | 1.2461 | 1.2962 |
| **Tyr** | **0.1754** | **0.2494** | **0.3122** | **0.4563** | **0.4835** | **0.5875** | **0.6735** | **0.7511** | **0.8131** | **0.8632** |
| Met | 0.0742 | 0.1336 | 0.1729 | 0.2193 | 0.2242 | 0.3282 | 0.4142 | 0.4918 | 0.5538 | 0.6039 |
| Val | 0.2103 | 0.2542 | 0.2871 | 0.3274 | 0.3799 | 0.4839 | 0.5699 | 0.6475 | 0.7095 | 0.7596 |
| Trp | 0.1075 | 0.0966 | 0.1472 | 0.1922 | 0.1923 | 0.2963 | 0.3823 | 0.4599 | 0.5219 | 0.572 |
| **Phe** | **0.4747** | **0.5515** | **0.5855** | **0.6957** | **0.835** | **0.939** | **1.025** | **1.1026** | **1.1646** | **1.2147** |
| **Ile** | **0.1616** | **0.1905** | **0.1977** | **0.296** | **0.3846** | **0.4886** | **0.5746** | **0.6522** | **0.7142** | **0.7643** |
| **Leu** | **0.6684** | **0.7696** | **0.8** | **0.9175** | **1.1456** | **1.2496** | **1.3356** | **1.4132** | **1.4752** | **1.5253** |
| Lys | 0.2109 | 0.1895 | 0.187 | 0.5971 | 2.4552 | 2.7592 | 3.0452 | 3.1228 | 3.1848 | 3.2349 |
| **Total** | **4.0022** | **4.7146** | **5.2189** | **6.3783** | **9.1994** | **11.2715** | **13.0195** | **14.4163** | **15.5323** | **16.4345** |

## 9.13 Raw data of economic calculation

Table 9.5 Different of substrate concentrations were hydrolyzed by 2% Alcalase and 2.5% Flavourzyme (w/w) at T 50°C and initial pH 9.0 for 10 tons corn gluten meal (CGM) in 20,000 L batch reactor.

| S% / Kg | 2% | 4% | 6% | 8% | 10% | 12% |
|---|---|---|---|---|---|---|
| Corn gluten | 10,000.00 | 10,000.00 | 10,000.00 | 10,000.00 | 10,000.00 | 10,000.00 |
| Alcalase | 124.76 | 124.76 | 124.76 | 124.76 | 124.76 | 124.76 |
| Flavourzyme | 155.95 | 155.95 | 155.95 | 155.95 | 155.95 | 155.95 |
| NaOH | 196.00 | 196.20 | 197.73 | 269.00 | 280.40 | 280.86 |
| Water | 304,515.20 | 147,090.20 | 92,249.95 | 71,170.27 | 52,601.28 | 37,900.08 |
| **S% / DM** | **2%** | **4%** | **6%** | **8%** | **10%** | **12%** |
| Corn gluten | 12,500.00 | 12,500.00 | 12,500.00 | 12,500.00 | 12,500.00 | 12,500.00 |
| Alcalase | 4,865.64 | 4,865.64 | 4,865.64 | 4,865.64 | 4,865.64 | 4,865.64 |
| Flavourzyme | 11,696.25 | 11,696.25 | 11,696.25 | 11,696.25 | 11,696.25 | 11,696.25 |
| NaOH | 35 | 35 | 35 | 48.42 | 50.91 | 51.07 |
| Water | 164.43 | 79.43 | 50.81 | 38.43 | 28.40 | 25.46 |
| **Subtotal [VC]** | **29,261.32** | **29,200** | **29,147.70** | **29,148.74** | **29,141.20** | **29,138.42** |
| Machine cost [1] | (20×13.64) | (10×13.64) | 6×13.64 | (5×13.64) | (4×13.64) | 3×13.64 |
| **Sum (DM)** | **272.80** | **130.64** | **81.84** | **68.20** | **54.56** | **40.92** |
| **Personal cost** [2] | (20×8) | (10×8) | (6×8) | (5×8) | (4×8) | (3×8) |
| DM/h | 26 | 26 | 26 | 26 | 26 | 26 |
| **Sum (DM)** | **4,160.00** | **2,080.00** | **1,386.32** | **1,040** | **832** | **692.15** |
| Insurance cost | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 |
| **Sum (DM)** | **7,072** | **3,536** | **2,356.74** | **1,768.00** | **1,414.40** | **1,176.66** |
| **Subtotal [FC]** | **7,344.80** | **3,666.64** | **2,438.58** | **1,836.20** | **1,468.96** | **1,217.57** |
| **Total[FC+VC] 8h** | **36606.12** | **32,866.64** | **31,586.28** | **30,984.94** | **30,610.16** | **30,356.00** |
| 4h | 35,568.27 | 31,877.37 | 30,611.72 | 30,038.38 | 29,664.03 | 29,597.19 |
| 14h | 53,660.17 | 40,923.32 | 36,640.86 | 34,561.36 | 33,282.41 | 32,609.49 |
| 24h | 71,652.17 | 49,919.32 | 42,636.69 | 39,059.36 | 36,880.81 | 35,605.16 |
| **Revenue** 4h | 31,808.20 | 29,039.97 | 32,616.39 | 32,336.52 | 32,502.20 | 30,533.66 |
| **8h** [3] | **44,757.30** | **40,627.30** | **40,348.19** | **41,156.53** | **42,120.21** | **42,204.30** |
| 14h | 59,619.10 | 52,430.90 | 48,778.30 | 50,242.70 | 51,616.90 | 53,927.50 |
| 24h | 66,865.80 | 65,540.80 | 56,057.50 | 57,712.96 | 58,419.70 | 61,787.70 |
| **Profit** 4h | -3,760.07 | -2,837.40 | 2,004.67 | 2,298.14 | 2,838.17 | 936.47 |
| **8h** [3] | **8,151.18** | **7,760.66** | **8,761.91** | **10,171.59** | **11,510.05** | **11,848.30** |
| 14h | 5,958.93 | 11,507.58 | 12,137.44 | 15,681.34 | 18,334.49 | 21,318.01 |
| 24h | -4,786.37 | 15,621.48 | 13,420.81 | 18,653.60 | 21,538.89 | 26,182.54 |

**(Profit = Revenues – Expenditure)**
**FC** = fixed cost (not vary with the level of output)
**VC** = variable cost

[1] a machine purchased for 90,000 DM, and the depreciation cost of this machine by straight-line method at 9,000 DM per year long life for 10 years [27.27 DM per day or 13.635 DM per cycle(8h)] but unconsidered energy costs, maintenance and repair costs.

[2] a persons for one machine multiply by hours of one cycle

[3] hours per cycle

**Revenue:** Try, Phe, Ile, Leu (prices: 37, 67, 95 and 43 respectively)

Table 9.6 Different of enzyme-substrate ratio between Alcalase and Flavourzyme [1 to 1], which hydrolyzed corn gluten meal at 8% protein (w/w), 50°C and initial pH 9.0 for 10 tons corn gluten meal in batch reactor volume 20,000 L .

| Kg \ E/S% | 3% | 3.7% | 5% |
|---|---|---|---|
| Corn gluten | 10,000.00 | 10,000.00 | 10,000.00 |
| Alcalase | 94.40 | 116.65 | 157.40 |
| Flavourzyme | 94.40 | 116.65 | 157.40 |
| NaOH | 125.30 | 125.30 | 125.30 |
| Water | 68,428.20 | 68,391.10 | 68,322.30 |
| **DM \ E/S%** | **3%** | **3.7%** | **5%** |
| Corn gluten | 12,500.00 | 12,500.00 | 12,500.00 |
| Alcalase | 2,832.00 | 3,495.00 | 4,722.00 |
| Flavourzyme | 7,131.90 | 8,801.50 | 11,891.50 |
| NaOH | 22.50 | 22.50 | 22.50 |
| Water | 36.90 | 36.90 | 36.90 |
| **Subtotal [VC]** | **22,523.30** | **24,855.90** | **29,172.90** |
| Machine cost [1] | (5×13.64) | (5×13.64) | (5×13.64) |
| **Sum (DM)** | **68.20** | **68.20** | **68.20** |
| Personal cost [2] | (5×8) | (5×8) | (5×8) |
| DM/h | 26.00 | 26.00 | 26.00 |
| **Sum (DM)** | **1,040** | **1,040** | **1,040** |
| Insurance cost | 1.7 | 1.7 | 1.7 |
| **Sum (DM)** | **1,768** | **1,768** | **1,768** |
| **Subtotal [FC]** | **1,836.20** | **1,836.20** | **1,836.20** |
| **Total[FC+VC]8h** | **24,359.50** | **26,692.10** | **31,009.10** |
| 4h | 24,330.83 | 26,663.43 | 30,980.43 |
| 14h | 28,853.82 | 31,186.40 | 35,503.40 |
| 24h | 33,351.80 | 35,684.40 | 40,001.40 |
| **Revenue** 4h | 20,196,60 | 25,881.40 | 34,009.20 |
| **8h** [3] | **31,773.60** | **36,152.30** | **44,286.80** |
| 14h | 35,675.60 | 45,522.50 | 50,926.60 |
| 24h | 46,051.10 | 48,693.30 | 58,281.40 |
| **Profit** 4h | -4,134.23 | -782.03 | 3,028.77 |
| **8h** [3] | **7,414.10** | **9,460.20** | **13,277.70** |
| 14h | 6,821.80 | 14,336.10 | 15,423.20 |
| 24h | 12,699.30 | 13,008.90 | 18,280.00 |

**(Profit = Revenues – Expenditure)**
**FC** = fixed cost (not vary with the level of output)
**VC =** variable cost

[1,2,3] see Table 9.5
**Revenue:** Try, Phe, Ile, Leu (prices: 37, 67, 95 and 43 respectively)

* 1 Euro = 1.95583 DM (01.07.2002)
1 Euro = 0.95586 US$ (01.07.2002)
1 US$ = 1.86950 DM (01.07.2002)

Table 9.7 Economic cost of enzymatic hydrolysis of corn gluten 10 tons dry wt.. Conditions; substrate conc.8% protein (w/w)], enzyme conc. 2% Alcalase2,4L and 2.5% Flavourzyme (w/w), 50°C, initial pH 9, pretreatment by heating at 80°C for 2 h prior to hydrolysis in 20 $m^3$ reactor.

| Kg \ h/cycle | 2h | 4h | 6h | 8h | 10h | 12h | 22h | 24h |
|---|---|---|---|---|---|---|---|---|
| Corn gluten | 10,000.00 | 10,000.00 | 10,000.00 | 10,000.00 | 10,000.00 | 10,000.00 | 10,000.00 | 10,000.00 |
| Alcalase | 124.76 | 124.76 | 124.76 | 124.76 | 124.76 | 124.76 | 124.76 | 124.76 |
| Flavourzyme | 155.95 | 155.95 | 155.95 | 155.95 | 155.95 | 155.95 | 155.95 | 155.95 |
| NaOH | 269.00 | 269.00 | 269.00 | 269.00 | 269.00 | 269.00 | 269.00 | 269.00 |
| Water | 71,170.27 | 71,170.27 | 71,170.27 | 71,170.27 | 71,170.27 | 71,170.27 | 71,170.27 | 71,170.27 |
| **DM \ h/cycle** | 2h | 4h | 6h | 8h | 10h | 12h | 22h | 24h |
| Corn gluten | 12,500.00 | 12,500.00 | 12,500.00 | 12,500.00 | 12,500.00 | 12,500.00 | 12,500.00 | 12,500.00 |
| Alcalase | 4,865.64 | 4,865.64 | 4,865.64 | 4,865.64 | 4,865.64 | 4,865.64 | 4,865.64 | 4,865.64 |
| Flavourzyme | 11,696.25 | 11,696.25 | 11,696.25 | 11,696.25 | 11,696.25 | 11,696.25 | 11,696.25 | 11,696.25 |
| NaOH | 48.42 | 48.42 | 48.42 | 48.42 | 48.42 | 48.42 | 48.42 | 48.42 |
| Water | 38.43 | 38.43 | 38.43 | 38.43 | 38.43 | 38.43 | 38.43 | 38.43 |
| **Subtotal [VC]** | **29,148.74** | **29,148.74** | **29,148.74** | **29,148.74** | **29,148.74** | **29,148.74** | **29,148.74** | **29,148.74** |
| Cycle/day | 8 | 4 | 3 | 2 | 2 | 1 | 1 | 1 |
| Machine cost[1] | (5×3.41) | (5×6.82) | (5×9.09) | (5×13.64) | (5×13.64) | (5×27.27) | (5×27.27) | (5×27.27) |
| **Sum(DM)** | **17.05** | **34.1** | **45.45** | **68.20** | **68.20** | **136.35** | **136.35** | **136.35** |
| Personal cost[2] | 5×2 | 5×4 | 5×6 | 5×8 | 5×10 | 5×12 | 5×22 | 5×24 |
| DM/h | 26 | 26 | 26 | 26 | 26 | 26 | 26 | 26 |
| **Sum (DM)** | 260 | 520 | 780 | 1,040 | 1,300 | 1560 | 2,860 | 3,120 |
| Incidental expenses | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 | 1.7 |
| **Sum(DM)** | 442 | 884 | 1,326 | 1,768 | 2,210 | 2,652 | 4,862 | 5,304 |
| **Subtotal [FC]** | **459.05** | **918.10** | **1,371.45** | **1,836.20** | **2,278.20** | **2,788.35** | **4,998.35** | **5,440.35** |
| **Total[VC + FC]** | **29,607.79** | **30,066.84** | **30,520.19** | **30,984.94** | **31,426.94** | **31,937.09** | **34,147.09** | **34,589.09** |
| **Revenues*** non | 20,861.14 | 30,914.19 | 35,524.54 | 41,156.53 | 45,425.17 | 49,030.12 | 53,705.75 | 52,770.52 |
| pret[3] | 20,529.04 | 32,281.73 | 37,423.47 | 43,569.90 | 47,131.25 | 52,417.02 | 54,727.67 | 53,938.70 |
| (+2h) pret | ***** | 20,529.04 | 32,281.73 | 37,423.47 | 43,569.90 | 47,131.25 | 52,417.02 | 54,727.67 |
| **Profit/cycle** non | -8,746.65 | 847.35 | 5,004.35 | 10,171.59 | 13,998.23 | 17,093.03 | 19,558.66 | 18,181.43 |
| pret | -9,078.75 | 2,214.89 | 6,903.28 | 12,584.96 | 15,704.31 | 20,479.93 | 20,580.58 | 19,349.61 |
| (+2h) pret | ***** | -9,537.80 | 1,761.54 | 6,438.53 | 12,142.96 | 15,194.16 | 18,269.93 | 20,138.58 |
| **Profit/day** non | -69,973.20 | 3,389.40 | 15,013.05 | 20,343.18 | 27,996.46 | 17,093.03 | 19,558.66 | 18,181.43 |
| pret | -72,630.00 | 8,859.56 | 20,709.84 | 25,169.92 | 31,408.62 | 20,479.93 | 20,580.58 | 19,349.61 |
| (+2h) pret | ***** | -38,151.20 | 5,284.62 | 19,315.59 | 24,285.92 | 15,194.16 | 18,269.93 | 20,138.58 |

**Profit = Revenues – Expenditure**
**FC** = fixed cost (not vary with the level of output)
**VC =** variable cost

[1] a machine purchased for 90,000 DM, and the depreciation cost of this machine by straight line method at 9,000 DM per year long life for 10 years [27.27 DM per day or 13.635 DM per cycle(8h)] but unconsidered energy costs, maintenance and repair costs (5×13.635).

[2] 5 persons for 5 machines multiply by hour number of a cycle and incidental expenses 1.7 factor (5×8x26x1.7).

[3] pretreatment with soluble heating at 80°C, pH 9.0 for 2 hours prior hydrolysis.

***Revenue:** Try, Phe, Ile, Leu (price: 37, 67, 95, 43 DM/kg respectively)

## 9.14 Mass balance

Table 9.8 Comparison data between acid hydrolysis and enzymatic hydrolysis of corn gluten meal and soybean meal.

| Amino acids [kg/t] | Corn gluten meal | | Soy protein meal | |
|---|---|---|---|---|
| | acid hydrolysis | enzymatic hydrolysis | acid hydrolysis | enzymatic hydrolysis |
| Asp | 32.56150 | 1.1714 | 48.2642 | 2.2212 |
| Glu | 115.0232 | 4.059 | 74.6252 | 5.8464 |
| Asn | -- | 10.2847 | -- | 5.6278 |
| Ser | 28.4030 | 10.072 | 22.2247 | 4.8081 |
| Gln | -- | 40.866 | -- | 4.1306 |
| His | 7.09675 | 3.339 | 7.44075 | 3.1877 |
| Gly | 12.0837 | 7.1781 | 16.0525 | 2.2893 |
| Thr | 14.2542 | 11.7317 | 13.4475 | 4.7927 |
| Arg | 48.3967 | 11.0807 | 19.4110 | 1.171 |
| Ala | 16.3432 | 18.0654 | 31.3887 | 3.8548 |
| **Tyr** | **27.0805** | **17.2663** | **14.3990** | **5.6918** |
| Met | 10.0302 | 8.7006 | 4.78625 | 3.157 |
| Val | 24.3380 | 16.05 | 18.5420 | 6.0688 |
| Trp | -- | 1.8641 | -- | 3.0147 |
| **Phe** | **34.0680** | **20.2657** | **22.0600** | **9.3346** |
| **Ile** | **22.8675** | **13.8446** | **19.8445** | **5.7616** |
| **Leu** | **97.5212** | **57.3293** | **33.5022** | **12.7901** |
| Lys | 13.5890 | 8.9477 | 40.7657 | 6.6379 |
| **Total** | **503.6575** | **262.1163** | **386.7550** | **90.3861** |
| **DH%** | **80** | **45** | **77** | **20** |

Table 9.9 Mass balance for ***acid*** hydrolysis of corn gluten meal. After 24 h of hydrolysis obtained 80% DH.

| Input | | | Output | | |
|---|---|---|---|---|---|
| substance | quantity [kg] | specification | substance | quantity [kg] | specification |
| corn gluten meal | 10,000 | solid | amino acids<br><br>target amino acids<br>other amino acids | 5,036.57<br><br>1,815.37<br>3,221.20 | liquid, fraction<br><br>(Phe,Ile,Leu,Tyr) |
| HCl[a] | 8,820 | liquid, 32% | NaCl | 5,511 | = 81% (60/74 % of potato protein[b]) |
| water[a] | 6,680 | | waste water | 20,153 | = 81% (60/74% of potato protein[b]) |
| NaOH[a] | 9,938 | liquid | residue (ash, residual solid substances) | 4,737.43 | |
| sum | 35,438 | | sum | 35,438 | |

[a] reference: Dr. Faurie (Amino GmbH); [1.3 M HCl/ mol peptid bond]

[b] assumption

Table 9.10 Mass balance for ***enzymatic*** hydrolysis of corn gluten meal. Conditions: substrate conc.: 8% protein (w/w) for 10 tons, enzyme conc.: 2% Alcalase and 2.5% Flavourzyme E/S% (w/w). After 24 h of hydrolysis obtained 45% DH.

| Input | | | Output | | |
|---|---|---|---|---|---|
| substance | quantity [kg] | specification | substance | quantity [kg] | specification |
| corn gluten meal | 10,000 | solid | amino acid<br><br>target amino acids:<br><br>other amino acids | 2,621.16<br><br>1,087.06<br><br>1,534.10 | liquid, fraction<br><br>(Phe,Ile,Leu,Tyr) |
| Alcalase | 124.76 | liquid | residue (animal feed) | 4,750.00 | solid |
| Flavourzyme | 155.95 | liquid | other fraction (peptide) | 2,628.84 | liquid |
| NaOH | 269.00 | | waste water | 71,000 | |
| water | 71,170.27 | | residue | 719.98 | |
| sum | 81,719.98 | | sum | 81,719.98 | |

Table 9.11 Mass balance for ***acid*** hydrolysis of soybean meal. After 24 h of hydrolysis obtained 77% DH.

| Input | | | Output | | |
|---|---|---|---|---|---|
| substance | quantity [kg] | specification | substance | quantity [kg] | specification |
| soybean meal | 10,000 | | amino acids<br><br>target amino acids<br><br>other amino acids | 3,867.55<br><br>898.06<br><br>296.95 | liquid, fraction<br><br>,(Ph,Ile,Leu,Tyr) |
| HCl[a] | 7,365.89 | liquid, 32% | NaCl | 4,597.30 | = 68% (50/74% of potato protein[b]) |
| water[a] | 5,729.01 | | waste water | 16,811.42 | = 68% (50/74% of potato protein[b]) |
| NaOH[a] | 8,299.97 | liquid | residue (ash, residual solid substances) | 6,118.60 | |
| sum | 31,394.87 | | sum | 31,394.87 | |

[a] reference: Dr. Faurie (Amino GmbH); [1.3 M HCl/ mol peptid bond]

[b] assumption

Table 9.12 Mass balance for ***enzymatic*** hydrolysis of soybean meal. Conditions: substrate conc.: 8% protein (w/w) for 10 tons, enzyme conc.: 2% Alcalase and 2.5% Flavourzyme E/S% (w/w). After 24 h of hydrolysis obtained 20% DH.

| Input | | | Output | | |
|---|---|---|---|---|---|
| substance | quantity [kg] | specification | substance | quantity [kg] | specification |
| soybean meal | 10,000 | solid | amino acid<br><br>target amino acids<br><br>other amino acids | 903.86<br><br>335.78<br><br>568.08 | liquid, fraction<br><br>(Phe,Ile,Leu,Tyr) |
| Alcalase | 99.75 | liquid | residue (animal feed) | 6,500.00 | solid |
| Flavourzyme | 122.91 | liquid | other fraction (peptide) | 2,596.14 | liquid |
| NaOH | 120.55 | liquid | waste water | 53,309.00 | |
| water | 53,899.16 | | residue | 442.86 | |
| sum | 63,266.14 | | sum | 63,266.14 | |

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