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MICROBIAL PROTEASES: A REVIEW

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ABSTRACT

This review provides an updated overview on the classification and important applications of the proteases more particularly microbial proteases. In recent years there has been an increase in demand of microbial proteases as industrial catalysts. Proteases of commercial importance are produced from microbial, animal and plant sources. Microbes are a preferred source of these enzymes because of their rapid growth, limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for various applications. The production of enzyme is central to modern biotechnology industry. Proteases constitute one of the most important group of industrial enzymes being extensively used in the food, pharmaceutical, protein hydrolysis, detergent, cheese-making, brewing, photographic, baking, meat, leather industries, inclusions in animal and human food as digestive aids, etc.

KEYWORDS: This review provides classification and important etc.

INTRODUCTION

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming part of metabolic processes of cells (Mohammad et al., 2013). Proteases, one of the largest group of industrial enzymes which accounts for about 59% of the global market of industrial enzymes (Deng *et al*., 2010).

Proteases which include proteinases, peptidases or proteolytic enzymes that break peptide bonds between amino acids of proteins. To produce environment friendly products chemical processes are being replaced by enzymes like proteases (Abebe *et al*., 2014). The production of enzyme is central to modern biotechnology industry. The technology for producing and

using commercially important enzyme products combine the discipline of microbiology, genetics, biochemistry and engineering.

Biologically active enzymes may be extracted from any living organism. A wide range of sources are used for commercial enzyme production from *Actinoplanes* to *Zymomonas*, from spinach to snake venom. More than hundred of enzymes used industrially, over halves are from fungi and yeast and over a third are from bacteria with the rest divided between animal (8%) and plant (4%) sources. A larger number of enzymes find use in chemical analysis and clinical diagnosis (Chaplin, 2004; Oyeleke *et al*., 2012). Proteases may play a vital role in chemical treatments by replacing the hazardous chemicals especially involved in soaking, dehairing and bating (Puvankrishnan and Dhar, 1986).

Enzymes produced from animal source includes trypsin, chymotrypsin, renin and pepsin. From plant source includes papain, bromelain, ficin, amylase and from microbes include subtilisin, proteinase K, collagenase and lipase. Microbes serve as a preferred source of these enzymes because of their rapid growth, limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for various applications (Kocher and Mishra, 2009).

Based on their acid behaviour, proteases are classified into three groups, that is, acid, neutral and alkaline proteases. Acid proteases perform best at pH range of 2.0-5.0 and are mostly produced by fungi (Aguilar *et al*., 2008).

Proteases having pH optima in range of 7.0 or around are called neutral proteases. Neutral proteases are mainly of plant origin while proteases having optimum activity at pH range of 8 and above are classified as alkaline proteases (Alnahdi., 2012).

Alkaline proteases produced from microorganisms play an important role in several industries e.g. tanning, photographic industries and waste treatments, etc (Gupta *et al*., 2002).

Screening of proteases producing Bacillus sp. from different ecological environments can result in isolation of new alkaline proteases with unique physiochemical characteristics (Singh *et al*., 1999).

Proteases

Proteases (EC 3.4.21-24 and 99; peptidyl -peptide hydrolases) are enzymes that hydrolyse proteins via the addition of water across peptide bonds and catalyse peptide synthesis in organic solvents and in solvents with low water content (Beg, 2003). The hydrolysis of peptide bonds by proteases is termed as proteolysis; the products of proteolysis are protein and peptide fragments, and free amino acids. Proteolytic enzymes are ubiquitous in occurrence, found in all living organisms, and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta, 2002). Proteases represent one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market accounting for about 60% of total worldwide sale of enzymes (Rao, 1998; Cowan, 1994). This dominance of proteases in the industrial market is expected to increase further by the year 2005 (Gupta, 2002b). The estimated value of the worldwide sales of industrial enzymes was $1 billion in 1998 (Rao, 1998). Proteases the most important group of enzymes produced commercially are used in detergent, protein, brewing, meat, photographic, leather and dairy industries (Anwar, 1998). Proteases have a long history of application in the food and detergent industries. Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals, is a relatively new development and has conferred added biotechnological importance (Rao, 1998). These enzymes have also become widely used in the detergent industry, since their introduction in 1914 as detergent additives (Gupta, 2002b). Proteases of commercial importance are produced from microbial, animal and plant sources. They constitute a very large and complex group of enzymes with different properties of substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles. Industrial proteases have application in a range of process taking advantage of the unique physical and catalytic properties of individual proteolytic enzyme types (Ward, 1991). This vast diversity of proteases, in contrast to the specificity of their action has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Rao, 1998).

2.1. Classification of Proteases

Proteases belong to a very large and complex group of enzymes which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima

and stability profile. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (Rao, 1998). Proteases can be classified according to 3 major criteria. Such as; i) the reaction catalysed ii) the chemical nature of the catalytic site, iii) the evolutionary relationship, as revealed by the structure. Proteases are broadly classified as endo or exoenzymes on the basis of their site of action on protein substrates. They are further categorized as serine proteases, aspartic proteases, cysteine proteases or metallo proteases depending on their catalytic mechanism (Geethanjali and Subash, 2011). They are also classified into different families and class depending on their amino acid sequences and evolutionary relationships. Based on the pH of their optimal activity, they are referred to as acidic, neutral, or alkaline proteases (Rao, 1998).

2.1.1. Exoproteases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases.

2.1.1.1. Aminopeptidases

Aminopeptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. They are known to remove the N-terminal amino acid methionine that may be found in heterogeneously expressed proteins but not in many naturally occurring mature proteins. Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi.

2.1.1.2. Carboxypeptidases

The carboxypeptidases act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallocarboxypeptidases and cysteine carboxypeptidases based on the nature of the amino acid residue at the active site of the enzymes (Rao, 1998).

2.1.2. Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group has the negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism (i) serine proteases (ii) aspartic proteases (iii) cysteine and (iv) metalloproteases (Pushpam *et al*., 2011). They are

also isolated from alfalfa, oats and barley senesced leaves which are involved in the process of protein degradation during foliar senescence (Nieri B.*et.al*, 1998), (Miller B.l. *et al*, 1981), (Drivdahl R.H. *et.al*, 1977).

2.2 Sources of proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, found in a wide diversity of sources such as plants, animals and microorganisms. Fortunately, enzymes can be separated from living cells and perform catalysis independent of the physiological environment. Commercial proteases are derived from animal tissues, plant cells and microbial cells via fermentation.

2.2.1 Plant Proteases

The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process. Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin (Rao, 1998). Papain, plant protease with a long history of use especially in tonics, which is active between pH 5 and 9 (Schechler and Berger, 1967). Papain and ficin are prepared by water extraction of crude material from *Carica papaya* and *Ficus carica* respectively. Bromelain is prepared from the stem and juice of pineapples. Bromelain is usually obtained from the stems of the pineapple plant by extraction and fractional solvent precipitation (Ward, 1985). Bromelain exerts anti-inflammatory effects in an ovalbumin-induced murine model of allergic airway disease (Secor *et al*., 2005). Bromelain and papain are plant-derived proteases with a longstanding history of use in a diverse range of food applications. As plant-derived products, they are perceived as safe and “natural” ingredients for use in the food applications and may offer unique benefits and functionality. The main problem with bromelain and papain are usually not cost effective.

2.2.2. Animal Proteases

The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin and rennin. These are prepared in pure form in bulk quantities. However their production depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies. Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins (Rao, 1998). Chymotrypsin is found in animal pancreatic extract. Pure chymotrypsin is an expensive enzyme and is used only for diagnostic and analytical

applications. Pepsin is an acidic protease that is found in the stomach of almost all vertebrates. Pepsin is prepared from the fundus portion of hag stomach, by acid extraction and filtration (Ward, 1985). Pepsin was used in laundry detergents as early as 1913, but is now being replaced by a mixture of serine and metal microbial proteases that appear to be less degradable by soaps, alkaline conditions and high temperatures (Adinarayana and Ellaiah, 2002). Rennet is a pepsin-like protease that is produced as an inactive precursor in the stomachs of all nursing mammals. It is converted to active rennin by the action of pepsin. It is used extensively in the dairy industry to produce a stable curd with good flavour.

2.2.3. Microbial Proteases

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Proteases of bacteria, fungi and viruses are increasingly studied due to its importance and subsequent applications in industry and biotechnology. Commercial application of microbial proteases is attractive due to the relative ease of large-scale production as compared to proteases from plant and animals. Microbial proteases account for approximately 40% of the total worldwide enzyme sales. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they posses almost all the characteristics desired for their biotechnological applications.

Microorganisms represent an attractive source of proteases as they can be cultured in large quantities in a relatively short time by established fermentation methods producing an abundant, regular supply of the desired product. Besides they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications (Gupta., 2002a). In general microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals (Gupta., 2002a). Microbial proteases, especially from *Bacillus sp.* have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in detergent formulations (Beg, 2003).

2.3. Alkaline Proteases

Alkaline proteases (EC.3.4.21-24, 99) are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine centre (serine protease) or are of metallo-type(metalloprotease); and they are the most important group of enzymes exploited commercially (Gupta., 2002b). Alkaline proteases are most active at pH values of about pH

10. They are sensitive to a potato inhibitor but not to TLCK or tosyl-L-phenylalanine chloromethyl ketone (TPCK). They are all specific against aromatic or hydrophobic amino acid residues at the carboxyl side of the splitting point (Ward, 1985). These enzymes also offer advantages over the use of conventional chemical catalysts for numerous reasons. For example they exhibit high catalytic activity, a high degree of substrate specificity, can be produced in large amounts and are economically viable (Anwar., 1998). Alkaline proteases being a physiologically and commercially important group of enzymes are used primarily as detergent additives. These enzymes have broad substrate specificities and will function to some extent under the rather extreme conditions encountered in domestic washing temperatures of 20 to 70ºC, a pH up to 11 and at high concentrations of detergents, polyphosphates, chelating agents such as EDTA and oxidizing agents such as sodium perborate (Cowan, 1994).

In recent years there has also been a phenomenal increase in the use of alkaline protease as industrial catalysts. In Japan, 1994 alkaline protease sales were estimated at $116 million. There is expected to be an upward trend in the use of alkaline proteases so that by the turn of the decade the total value for industrial enzymes is likely to reach 700 million or more (Kumar and Takagi, 1999).

Especially, alkaline proteases are of microbial origin, which dominate the worldwide enzyme market, possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Agrawal., 2004, Gupta., 2002a). Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues (Singh., 2001a). Despite their interest in other microbial sources, survey of the literature conclusively shows that bacteria are by far the most popular source of commercial alkaline proteases to date. Important alkaline proteases are solanain, hurain and proteolytic enzymes of Bacillus and Streptomyces species (Hameed *et al*., 1996; Lee *et al*., 2002; Tang *et al*., 2004).

Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10 and their broad substrate specificity. Their optimal temperature is around 60ºC. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao., 1998).

From all the alkaliphilic bacteria that have been screened for use in various industrial applications, members of the genus Bacillus, mainly strains *B.subtilis* and *B.licheniform* were found to be predominant and a prolific source of alkaline proteases (Kumar and Takagi, 1999).

2.4. Industrial Applications of Alkaline Proteases from Bacillus Strains

They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar *et al*., 2007). They have been routinely used for various purposes such as cheese making, baking, preparation of soya hydrolysates and meat tenderization. The major application of protease in the dairy industry is in the manufacture of cheese. Milk contains proteins, specifically caseins that maintain its liquid form. Proteases are enzymes that are added to milk during cheese production, to hydrolyze caseins, specifically kappa casein, which stabilizes micelle formation preventing coagulation. Rennet and rennin are also used to coagulate milk. Technically rennet is also the term for the lining of a calf's fourth stomach. The most common enzyme isolated from rennet is chymosin. Chymosin can also be obtained from several other animal, microbial or vegetable sources, but indigenous microbial chymosin (from fungi or bacteria) is ineffective for making cheddar and other hard cheese (Sawant and Nagendran, 2014).

2.4.1. Food Industry

Alkaline proteases can hydrolyse proteins from plants and animals to produce hydrolysates of well-defined peptide profiles. These protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations specific therapeutic dietary products and the fortification of fruit juices and soft drinks. In recent years there has been substantial interest in developing enzymatic methods for the hydrolysis of soya protein, gelatin, casein, whey and other proteins in order to prepare protein hydrolysates of high nutritional value. In developing commercial products from these proteins, emphasis is placed on achieving a consistent product in high yields, having desirable flavour, nutritional and/or functional properties (Ward, 1991).

Alkaline protease from *B.licheniformis* is used for the production of highly functional protein hydrolysates (Ward, 1991). This commercial alkaline protease, Alcalase, has a broad specificity with some preference for terminal hydrophobic amino acids. Using this enzyme, a less bitter hydrolysate and a debittered enzymatic whey protein hydrolysate were produced (Kumar and Takagi, 1999). Soluble meat hydrolysate can also be derived from lean meat

wastes and from bone residues after mechanical deboning by solubilization with proteolytic enzymes. Alcalase has been found to be the most appropriate enzyme in terms of cost, solubilization, and other relevant factors. In an optimized process with Alcalase at a pH of 8.5 and temperature of 55-60ºC, a solubilization of 94% was achieved. The resulting meat slurry was further pasteurized to inactivate the enzyme and found wide application in canned meat production, soups and seasoning (Kumar and Takagi, 1999). Very recently, another alkaline protease from *B. amyloliquefaciens* resulted in the production of a methionine-rich protein hydrolysate from chickenpea and soy protein, which found major application in hypoallergenic infant food formulations (Kumar and Takagi, 1999). In another study, Rebecca., (1991) reported the production of fish hydrolysate of high nutritional value, using *B. subtilis* proteases. Perea., (1993) on the other hand used alkaline protease for the production of whey protein hydrolysate, using cheese whey in an industrial whey bioconversion process.

2.4.2. Detergent Industry

Enzymes have long been of interest to the detergent industry for their ability to aid the removal of proteinaceous stains and to deliver unique benefits that cannot otherwise be obtained with conventional detergent technologies (Gupta., 2002b). The use of enzymes in detergent formulations is now common in developed countries, where more than half of the presently available ones contain enzymes (Chaplin and Bucke, 1990). Detergent enzymes account for approximately 30% of total worldwide enzyme production (Horikoshi, 1996) and 89% of the total protease sales in the world; a significant share of the market is captured by subtilisins and/or alkaline proteases from many *Bacillus* species (Gupta., 2002b). Ideally alkaline proteases used in detergent formulations should have high activity and stability over a broad range of pH and temperature, should be effective at low levels (0.4-0.8%) and should also be compatible with various detergent components along with oxidizing and sequestering agents. They must also have a long shelf life (Kumar and Takagi, 1999). Alkaliphilic Bacillus strains are good sources of alkaline proteases with the properties that fulfil essential requirements to be used in detergents; therefore the main industrial application of alkaliphilic proteases has been in the detergent industry since their introduction in 1914 as detergent additives. (Ito., 1998; Horikoshi, 1996). The major use of detergent–compatible proteases is in laundry detergent formulations. Detergents available in the international market such as Dynamo®, Eraplus® (Procter & Gamble), Tide®(Colgate Palmolive), contain proteolytic

enzymes, the majority of which are produced by members of the genus Bacillus (Anwar., 1998).

The main producers of alkaline proteases using species of Bacillus are the companies such as Novo Industry A.I.S. and Gist Brocades. Novo produces three proteases, Alcalase from *B. licheniformis*, Esperase from an alkalophilic strain of a *B. licheniformis* and Savinase from an alkalophilic strain of a *B. amyloliquefaciens*. Gist Brocades on the other hand produces and supplies Maxatase from *B.licheniformis*. Alcalase and Maxatase (both mainly subtilisin) are recommended to be used at 10-65ºC and pH 7-10.5. Savinase and Esperase can be used at up to pH 11 and 12 respectively (Chaplin and Bucke, 1990).

Conventionally, detergents have been used at elevated washing temperatures, but at present there is considerable interest in the identification of alkaline proteases, which are effective over wider temperature ranges (Oberoi., 2001). For example there is considerable current interest on the exploration of proteases that can catalyse reactions in cold water. This allows their use in detergents, which can be used in normal tap water without the requirement for increasing the temperature of the water. The search for such enzymes is very much a challenge at this time (Haki and Rakshit, 2003). Banerjee and his colleagues (1999) have studied on an alkaline protease from a facultative thermophilic and alkalophilic strain of *Bacillus brevis*. The alkaline protease from *Bbrevis* having maximum activity at pH 10.5, showed a high level of thermostability at 60ºC. The enzyme showed compatibility at 60ºC with all of the commercial detergents tested. It could also remove blood strains completely when used with detergents. All the tests were studied in the presence of Ca^{2+} and glycine and the data obtained in this study implies that the protease of *B. brevis* has most of the properties to be used as a detergent enzyme. In another study, Gupta and his friends (1999) reported a bleach-stable & thermotolerant alkaline protease from a new variant of *Bacillus sp*., having potential application in detergent formulations. The alkaline protease from newly isolated Bacillus SB5 displayed stability in the presence of 10% (v/v) H_2O_2 (oxidizing agent) and 1% SDS (sodium dodecyl sulphate, surfactant). The enzyme had an optimum activity at pH 10 and 60ºC to 70ºC, where this was further increased in the presence of all ionic and non-ionic detergents, surfactants and commercial detergents tested (Gupta., 1999). Oberoi and his colleagues (2001) also produced an alkaline, SDS-stable protease from *Bacillus sp*. RGR-14 that was also suggested to be an ideal detergent additive for detergent formulations. The enzyme was active over a wide temperature range in alkaline conditions. In addition being

SDS-stable it was also stable towards oxidizing agents such as H_2O_2 and sodium perborate. Protease and amylase are used particularly in dishwasher detergents to remove protein and carbohydrate containing food particles (Keshwani et al., 2015). Protease digests on organic stains, such as grass, blood, egg and human sweat, whereas cellulases are used to brighten colors, soften fabrics and to eliminate small fibers from the fabric without damaging the major fibers of the fabric (Hasan *et al*., 2010; Kuhad *et al*., 2011).

The alkaline protease from *B. clausii I-52* is significant for industrial perspective because of its ability to function in broad pH and temperature ranges in addition to its tolerance and stability in presence of an anionic surfactant like SDS and oxidants like peroxides and perborates. The enzymatic properties of this protease therefore suggest its suitable application as additive in detergent formulations (Joo., 2003).

2.4.3. Leather Industry

The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and safety hazards. Thus, for environmental reasons, biotreatment of leather using enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced (Gupta., 2002b). Alkaline proteases with elastolytic and keratinolytic activity can be used in leather processing industries. During bating, the hide is softened by partial degradation of the interfibrillar matrix proteins (elastin & keratin). Therefore enzyme preparations with low levels of elastase and keratinase activity but no collagenase activity are particularly applicable for this process (Cowan, 1994). Bating is traditionally an enzymatic process involving pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular. The substitution of chemical depilatory agents in the leather industry by proteolytic enzymes produced by *Bacillus sp*. could have important economical and environmental impacts (Anwar., 1998) where the dehairing process is accelerated by the use of alkaline proteases.

2.4.4. Medical usage

Alkaline proteases are also used for developing products of medical importance. It was stated in Gupta (2002b) that Kudrya and Simonenko, 1994 exploited the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of

burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kim., (2001) reported the use of alkaline protease from *Bacillus sp.* strain CK 11-4 as a thrombolytic agent having fibrinolytic activity (Gupta., 2002b). Furthermore, *Bacillus sp*. has been recognized as being safe to human (Kumar and Takagi, 1999).

2.4.5. Peptide Synthesis

Amino acids are of increasing importance as dietary supplements for both humans and domestic animals. Only the L-amino acids can be as similated by living organisms, since the chemical synthesis of amino acids produces a racemic mixture, it is necessary to separate the isomers before commercial use. Alcalase is a proteolytic enzyme isolated from a selected strain of B. licheniformis, its major component being subtilisin Carslberg. It was determined that Alcalase was stable in organic solvents and could be of use as a catalyst in the resolution of N-protected amino acids having unusual side chains (Anwar., 1998).

2.5.8. Textile Industry

One of the least explored areas for the use of proteases is in the silk industry where only a few patents have been filed describing the use of proteases for the degumming process of silk. The conventional silk degumming process is generally expensive and therefore an alternative method suggested, is the use of protease preparations for degumming the silk prior to dyeing. In a recent study, the silk degumming efficiency of an alkaline protease from *Bacillus sp. RGR-14* was studied. After 5h of incubation of silk fiber with protease from *Bacillus sp*., the weight loss of silk fiber was 7.5%. Scanning electron microscopy of the fibers revealed that clusters of silk fibers had fallen apart as compared with the smooth and compacted structure of untreated fiber (Gupta., 2002).

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