Microbial Enzymes and Biotechnology

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Preface

Biotechnology has its origins in antiquity. Many processes relating to production of foods have been practised for thousands of years, e.g. production of cheese, fermented milk products, beer, wine and vinegar. These fermentations developed from an art form and only recently have they evolved to more technically sophisticated processes, e.g. production of beer and vinegar. The discovery of antibiotics some forty years ago and the subsequent development of antibiotic fermentations was a major milestone in industrial microbiology and may be regarded as the most significant development in biotechnology in the first half of the twentieth century.

The large-scale production of microorganisms, under conditions of rigorous sterility and controlled nutritional and environmental conditions, permitted maximization of the production of these metabolites which formed the basis of a new antibiotic industry producing, for example, penicillin, cephalosporin, tetracyclines, etc. The fundamental knowledge acquired from the large-scale production of microorganisms under such conditions and the concurrent application of process engineering principles to this development formed the basis from which other microbiologically based industries evolved. The microbial enzyme industry is one example of such a development. Although microbial enzymes have been used for centuries in whole-cell processes, the commercial use of isolated microbial enzymes, on a large scale, received a major boost in the 1960s following the inclusion of alkaline proteases in washing powders. The great bulk of enzymes used indus-

trially are of microbial origin and with few exceptions, e.g. glucose isomerase, are produced extracellularly and are hydrolytic in character. In recent years other intracellular enzymes like penicillin acylase and asparaginase have been produced industrially. Industrial enzymology and the development of new enzymological processes embody one of the most significant areas of new growth in biotechnology. Therefore, it is essential that advancements in this field are reviewed and updated regularly. This text is aimed at fulfilling such a need as far as the enzymes of major significance are concerned.

I wish to express my gratitude and appreciation to my colleague Dr C. T. Kelly for help in the preparation of this book.

WILLIAM M. FOGARTY

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Chapter 1

Microbial Amylases

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1 INTRODUCTION

Amylases, starch-degrading enzymes, have numerous biotechnical applications, e.g. in the production of syrups containing oligosaccharides, maltose and glucose. The precise composition of the end-product can be controlled so that products with desired physical properties may be obtained (Palmer, 1975). Enzymatic degradation of starch on an industrial scale has been practised for many years and has replaced to a considerable extent the traditional acid-catalysed processes (Barfoed, 1976; Underkofler *et al.*, 1965). In the USA, over 75% of syrup and solid dextrose is now produced by enzymatic processes. New developments have taken place in the area of starch-degrading enzymes. Thermostable α-amylase from *Bacillus licheniformis* and the amylopectin debranching enzyme pullulanase, from *Aerobacter aerogenes*, have become available commercially. There is considerable interest in the commercial application of immobilized amyloglucosidase. Since the discovery of microbial β-amylases in this laboratory (Fogarty & Griffin, 1973, 1975; Griffin & Fogarty, 1973*a*, *b*) the commercial potential of this enzyme has stimulated interest in numerous laboratories throughout the world.

This chapter will review the more important microbial amylases from a technological viewpoint and summarize important attributes of more recently described enzymes. Earlier work has been reviewed by Fischer & Stein, 1960; Greenwood & Milne, 1968*a*; Fogarty & Kelly,

1979, 1980 and Norman, 1979. Reviews on the properties and chemistry of starch—the natural substrate for amylases—have been published elsewhere (Banks & Greenwood, 1975; Greenwood, 1976; Manners, 1974; Radley, 1976). Methods for the detection and assay of amylolytic enzymes are based on the use of starch or a derivative of starch as the substrate and have been outlined recently (Fogarty & Kelly, 1979). New chromogenic substrates have been developed recently for the quantitative assay of α-amylases (McCleary, 1980).

2 MICROBIAL AMYLASES

2.1 α-Amylase

2.1.1 *Mode of Action and Distribution*

α-Amylase (α-1,4-D-glucan glucanohydrolase, EC 3.2.1.1, endo-amylase) is distributed widely in microorganisms (Table 1). It hydrolyses α-1,4-glucosidic bonds in amylose, amylopectin and glycogen in an endo-fashion, but α-1,6-glucosidic linkages in branched polymers are not attacked. The properties and mechanisms of action of α-amylases depend on the source of the enzyme. They are all endo-acting enzymes, and therefore effect a rapid decrease in iodine-staining power and a simultaneous rapid decrease in the viscosity of starch solutions. Hydrolysis of amylose by α-amylase causes its conversion into maltose and maltotriose, initially. Hydrolysis of maltotriose, which is a poor substrate, follows in a second stage reaction (Walker & Whelan, 1960*a*). Hydrolysis of amylopectin by α-amylase also yields glucose and maltose in addition to a series of branched α-limit dextrins. These dextrins of four or more glucose residues contain all the α-1,6-glucosidic linkages of the original structure. With amylopectin or glycogen, the second stage of α-amylase degradation involves slow hydrolysis of maltotriose as well as slow hydrolysis of specific bonds near the branch points of the α-limit dextrins as, for example, the hydrolysis of 6^3-α-maltotriosyl maltotetraose to 6^3-α-glucosyl maltotetraose and maltose. Different α-amylases produce different α-limit dextrins (Whelan, 1960; Umeki & Yamamoto, 1975a, 1977). In addition to singly branched α-limit dextrins, it is highly probable that some α-amylases produce multiple-branched dextrins (French, 1960; Roberts & Whelan, 1960). Isomaltose is not formed in these reactions (Manners & Marshall, 1971) because α-1,6 linkages are resistant to

TABLE 1

DISTRIBUTION OF SOME MICROBIAL α-AMYLASES

Organism	*Reference*
Bacillus acidocaldarius	Buonocore *et al.* (1976)
Bacillus amyloliquefaciens	Welker & Campbell (1967*c*); Granum (1979)
Bacillus caldolyticus	Grootegoed *et al.* (1973); Heinen & Heinen (1972)
Bacillus coagulans	Bliesmer & Hartman (1973)
Bacillus licheniformis	Meers (1972); Saito & Yamamoto (1975); Chiang *et al.* (1979); Medda & Chandra (1980); Morgan & Priest (1981)
Bacillus stearothermophilus	Manning & Campbell (1961); Ogasahara *et al.* (1970*c*); Davis *et al.* (1980)
Bacillus subtilis	Bailey & Markkanen (1975); Kokubu *et al.* (1978); Matsuzaki *et al.* (1974*a*, *b*); Kennedy & White (1979)
Bacillus subtilis var. *amylosacchariticus*	Fujimori *et al.* (1978); Matsuno *et al.* (1978); Toda & Narita (1968)
Bacillus sp.	Srivastava *et al.* (1980)
Bacillus spp. (alkalophilic)	Boyer & Ingle (1972); Horikoshi (1971*a*); Yamamoto *et al.* (1972)
Bacteroides amylophilus	McWethy & Hartman (1977)
Clostridium acetobutylicum	Ensley *et al.* (1975)
Lactobacillus amylophilus	Nakamura & Crowell (1979)
Micrococcus halobius	Onishi & Sonoda (1979)
Streptomyces aureofaciens	Hostinova & Zelinka (1978)
Streptomyces hygroscopicus	Hidaka & Adachi (1980)
Streptomyces praecox	Suganuma *et al.* (1980)
Thermoactinomyces vulgaris	Allen & Hartman (1972); Shimizu *et al.* (1978)
Thermomonospora curvata	Stutzenberger & Carnell (1977)
Thermomonospora vulgaris	Allam *et al.* (1977)
Thermomonospora viridis	Upton & Fogarty (1977)
Acinetobacter sp.	Onishi & Hidaka (1978)
Thermophile V-2	Hasegawa *et al.* (1976)
Pseudomonas hydrogenovora	Igarashi *et al.* (1980)
Pseudomonas saccharophila	Markowitz *et al.* (1956)
Aspergillus awamori	Watanabe & Fukimbara (1967)
Aspergillus batatae	Bendetskii *et al.* (1974)
Aspergillus foetidus	Hang & Woodams (1977)
Aspergillus niger	Aski *et al.* (1971); Ramachandran *et al.* (1978)
Aspergillus oryzae	Vallier *et al.* (1977); Yabuki *et al.* (1977); Bata *et al.* (1978)
Aspergillus terricola	Aravina & Ponomarera (1977)

TABLE 1—*contd.*

Organism	*Reference*
Mucor miehei	Adams & Deploey (1976)
Mucor pusillus	Adams & Deploey (1976); Somkuti & Steinberg (1980)
Neurospora crassa	Gratzner (1972)
Paecilomyces subglobosum	Kim & Byun (1978)
Penicillium expansum	Belloc *et al.* (1975)
Lipomyces kononenkoae	Spencer-Martins & van Uden (1979)
Lipomyces starkeyi	Moulin & Galzy (1979)
Schwanniomyces castellii	Oteng-Gyang *et al.* (1981)
Torulopsis ingeniosa	Moulin & Galzy (1978*c*); Clementi *et al.* (1980)
Endomycopsis fibuligera	Clementi *et al.* (1980)

α-amylases and they confer some stability on certain α-1,4 linkages near the branch points. Formation of enzyme–substrate complexes appears to be restricted by the presence of these branch (α-1,6) linkages.

Bacillus amyloliquefaciens liquefying α-amylase produces singly branched dextrins from β-limit dextrin. They consist of not more than nine glucose units (Umeki & Yamamoto, 1977). The structure of one of these, a singly branched heptaose, was determined (Umeki & Yamamoto, 1975*a*). This dextrin was a mixture of six singly branched heptaoses with different branch points but they all contained a 6^2-α-maltosylmaltotriose unit in their structures. They differed only in the manner of linkage, by α-1,4 glucosidic bonds of two glucose units or one maltose residue, to the core dextrin.

Doubly branched limit dextrins are produced by saccharifying α-amylase of *Bacillus subtilis* (Umeki & Yamamoto, 1972). They all contain a 6^3-α-glucosylmaltotriose residue at the reducing end and an isomaltosyl residue at the non-reducing end. Two highly branched dextrins containing nine and ten glucose units were isolated and both were shown to be mixtures of four triply branched dextrins and were made up of 6^3-α-glucosylmaltotriose and/or 6^2-α-glucosylmaltotriose as a linking unit (Umeki & Yamamoto, 1975*b*).

α-Amylases from different sources have been purified or crystallized. Examples of purified preparations include those of various *Bacillus* spp., e.g. *B. subtilis* (Moseley & Keay, 1970; Yamamoto, 1955), *B. coagulans* (Campbell, 1954), *B. stearothermophilus* (Man-

ning & Campbell, 1961; Ogasahara *et al.*, 1970*a*; Pfueller & Elliott, 1969), *B. amyloliquefaciens* (Welker & Campbell, 1967*c*; Granum, 1979), *B. licheniformis* (Saito, 1973; Morgan & Priest, 1981); and other organisms, e.g. *Acinetobacter* sp. (Onishi & Hidaka, 1978), *Pseudomonas saccharophila* (Markowitz *et al.*, 1956), *Aspergillus oryzae* (Akabori *et al.*, 1954; Fischer & De Montmollin, 1951*a*, *b*; Toda & Akabori, 1963; Underkofler & Roy, 1951), *Aspergillus niger* (De Song Chong & Tsujisaka, 1976; Minoda & Yamada, 1963), *Micrococcus halobius* (Onishi & Sonoda, 1979), *Streptomyces hygroscopicus* (Hidaka & Adachi, 1980) and *Thermoactinomyces vulgaris* (Shimizu *et al.*, 1978). Polyacrylamide-gel electrophoresis has been used to demonstrate homogeneity of the crystalline α-amylases of *B. amyloliquefaciens* (Welker & Campbell, 1967*c*), *Bacillus stearothermophilus* (Pfueller & Elliot, 1969) and *A. oryzae* (McKelvy & Lee, 1969). Electrophoresis of purified α-amylase of *Bacillus licheniformis* (Saito, 1973) indicated that it contained four active protein bands although the enzyme behaved as a single peak during ultracentrifugation.

Because of their commercial and industrial uses α-amylases from many sources have been studied in great detail. The genus *Bacillus* is the single most important bacterial source of this enzyme. Of particular commercial significance, because of their great thermostability, are the enzymes produced by *B. amyloliquefaciens* and *B. licheniformis*.

2.1.2 *Liquefying and Saccharifying α-Amylases*

The bacilli produce saccharifying and liquefying α-amylases (Table 2) (Fukumoto, 1963; Welker & Campbell, 1967*a*, *b*). They are distinguishable by their mechanisms of starch degradation because the saccharifying α-amylase produces an increase in reducing power about twice that of the liquefying enzyme (Fukumoto, 1963; Pazur & Okada, 1966). *Bacillus subtilis* var. *amylosaccharitícus*, *B. subtilis* Marburg and *Bacillus natto* all produce saccharifying α-amylase (Matsuzaki *et al.*, 1974*b*; Yoneda *et al.*, 1974). *Bacillus amyloliquefaciens* produces large quantities of liquefying α-amylase (Welker & Campbell, 1967*a*, *b*).

On the basis of biochemical and physiological properties, Welker & Campbell (1967*a*) showed that highly amylolytic strains of *B. subtilis* were, in fact, *B. amyloliquefaciens*. These data confirmed the observation of Fukumoto (1943*a*), who classified the strains as *B. amyloliquefaciens* n.sp. Fukumoto (Fukumoto, 1943*a*, *b*). This organism was

TABLE 2

COMPARISON OF THE PROPERTIES OF *Bacillus subtilis* var. *amylosaccharitícus* (SACCHARIFYING α-AMYLASE) AND *Bacillus amyloliquefaciens* (LIQUEFYING α-AMYLASE) STARCH-DEGRADING ENZYMES

	B. *subtilis* var. amylosaccharitícus	B. amyloliquefaciens
pH optimum	6·8	5·9
Temperature optimum (°C)		70
Thermostability (50% inactivation, °C)	68	80
Hydrolysis limit on soluble starch (%)	43	16
Molecular weight	41 000	49 000
Isoelectric point		5·2
End-products	G_1, G_2	G_6
Adsorption on raw starch (%)	6	88
K_m (starch)	2·31 mg/ml	6·9 mg/ml

Abbreviations: G_1, glucose; G_2, maltose; G_6, maltohexaose.

described subsequently as *B. amyloliquefaciens* (Welker & Campbell, 1967*a*, *b*, *c*), a strain of *B. subtilis* (Hagihara, 1960) or *B. subtilis* var. *amyloliquefaciens* (Tsuru, 1962; Tsuru & Fukumoto, 1963). The bacterium producing the saccharifying enzyme is referred to as *B. subtilis* or *B. subtilis* var. *amylosaccharitícus* (Fukumoto, 1963). Authentic strains of *B. subtilis* produce α-amylases that differ immunologically and electrophoretically from strains of *B. amyloliquefaciens* (Welker & Campbell, 1967*b*).

Methods of crystallizing α-amylases of *B. amyloliquefaciens* have been reported (Welker & Campbell, 1967*c*). The enzymes from five strains are identical with regard to pH and temperature optima, UV absorption spectra, immunological and electrophoretic properties. However, the enzymes differ with respect to temperature and pH stabilities, activity on various substrates, K_m and energy of activation. These differences were not explained by structural alterations since it was shown (Borgia & Campbell, 1978) that the primary structure of the α-amylase of the five strains was identical or only possessed slight modifications. Almost identical compositions for the five α-amylases were established by amino acid analyses. The N-terminal amino acid of each amylase was valine and the C-terminal residue in each case was

tyrosine. Isoelectric points for the enzymes ranged between 5·09 and 5·18. These data, therefore, strongly support the view that the amylases from five strains of *B. amyloliquefaciens* are basically identical and strengthen the view that the five strains represent a species other than *B. subtilis*. The position regarding the alkaline proteases produced by these organisms is somewhat similar. Differences between subtilisin Novo (or BPN') and subtilisin Carlsberg in electrophoretic mobility were shown to be the result of differences in amino acid sequences (Smith *et al.*, 1966). *Bacillus subtilis* Novo and *B. subtilis* BPN' have been shown to be strains of *B. amyloliquefaciens* (Welker & Campbell, 1967*a*). The amino acid composition of the α-amylase of *B. amyloliquefaciens* (Borgia & Campbell, 1978) is not unlike that presented for the α-amylase of *B. subtilis* (*B. amyloliquefaciens* strain T) (Junge *et al.*, 1959), but it is different from that reported for α-amylase of authentic strains of *B. subtilis* (Yamane *et al.*, 1973). Furthermore, it had been shown in earlier work (Welker & Campbell, 1967*b*) that *B. subtilis* α-amylase does not cross react with antiserum prepared against *B. amyloliquefaciens* α-amylase and there appear to be differences between the structures of the two enzymes (Nagata *et al.*, 1980).

The saccharifying amylase of *B. subtilis* var. *amylosaccharíticus* produces more glucose from starch than the liquefying α-amylase of *B. amyloliquefaciens* (Fukumoto, 1963; Pazur & Okada, 1966), although the saccharifying enzyme does possess liquefying properties. Furthermore, it is immunologically distinct from the liquefying enzyme (Moseley & Keay, 1970) and, in contrast, possesses both cyclodextrinase (Moseley & Keay, 1970) and maltase (Yoshida *et al.*, 1969) activities. The rate of production of glucose from starch is much slower than the rate of production of reducing sugars (Keay, 1970). The enzyme does not function, therefore, as an amyloglucosidase, by exclusively hydrolysing glucose from the chain ends. Immunological methods demonstrated that the liquefying properties of the saccharifying enzyme were not due to a contaminating carbohydrase. Attempts to separate the amylase, cyclodextrinase and maltase activities of saccharifying amylase by inhibitors, electrophoresis, heat inactivation or chromatography were unsuccessful and it was concluded that the three activities were due to one molecular species (Keay, 1970).

2.1.3 *General Characteristics of α-Amylases*

α-Amylases are generally stable in the pH range 5·5–8·0 and to extremes of pH in the presence of a full complement of calcium. Optimal