Enzymology of the national microorganisms of Japan in a historical context.

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Abstract

Since the old days, Japanese people have produced alcoholic drinks using rice as the main raw material. Almost all the traditional fermented foods in Japan have been developed using with malted rice (*"kōji"* in Japanese) using *Aspergillus oryzae*. The year of 1894 saw the true beginning of modern microbial enzyme technology, with "Taka-diastase". Examples of major medicines that have been used continuously or more than 100 years include Adrenalin, Aspirin and Taka-diastase. Modern enzymology in Japan is advancing by quantum leaps over 100 years. All described in the text are widely recognized worldwide. After careful screening, a selected group of less than 100 references was chosen for a historical context. Gucoamylase, ribonuclease T1, and nuclease S1 are discovered, respectively. Many characteristic phenomena are also discovered with several enzymes. In 2006, the Brewing Society of Japan authorized the certification of "*Aspergillus oryzae, Aspergillus sojae* and *Aspergillus luchuensis* are as the national microorganisms (fungi) of Japan". Traditional Japanese cuisine ("*washyoku*" in Japanese) was added to the United Nations Educatuional, Scientific and Cultural Organaization's (UNESCO's) Intangible Culture Heritage list in 2013. Leading studies of enzymology have introduced new systems which are at the forefront of modern biotechnology.

Keywords: *Aspergillus luchuensis*; *Aspergillus oryzae*; *Aspergillus sojae*; *Kōji*; National microorganism; Traditional Japanese cuisine; UNESCO.

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Historical aspects of the national microorganisms

Thirteen poems in praise of wine by Lord Ōtomo Tabito,

Commander of the Dazaifu

What the Seven Wise Men

of ancient times

wanted, it seems

was wine.

Ōtomo Tabito (665-731). Man'yōsyū. Vol. 3. No. 340 [1].

Over the past 1,000 years, the use of hydrolytic enzymes from fungi has become more prevalent in Japanese fermentation industries. *Saké*, the glorious, national alcoholic drink of Japan, was born out of Japanese culture) [2]. Almost all the traditional fermented foods in Japan have been developed using with malted rice ("*kōji*") [3] in Japanese, Figure 1) using *Aspergillus oryzae*.

In 1932, Udo identified L-glutamate (monosodium glutamate) as the most important taste and flavor component ("*umami*") of soy sauce ("*shoyu*") [4], the Japanese traditional fermented food. As a specific taste receptor [5], the homeodomain protein, Skn-1a/Pou2f3, was identified in 2012. Functional diversification of taste receptor cells is critical for proper discernment of taste qualities. To make soy sauce ("*shoyu*") [6], the cooked soybeans are mixed with equal amounts of roasted wheat and then inoculated with a pure cultured *kōji* starter or seed mold. *Aspergillus sojae* is used to produce soy sauce ("*shoyu*") and fermented soybean paste ("*miso*") due to its high proteolytic quality. Fermented soybean paste ("*miso*") reduced fat in a dose-dependent manner and, inhibited hypertrophy of adipocyte in mice [7].

Figure 1: Japanese ideographic character "糀"("*kōji*" in Japanese). Explanation of the Japanese ideographic character "糀" ("*kōji*"): The left part of the Japanese ideographic character "糀" ("*kōji*" in Japanese) means rice (米, "*kome*" in Japanese), and the right part means "flower" (花, "*hana*" in Japanese). The ideographic characters ("*kokuji*") were created letters in Japan [3].

The *Aspergillus oryzae* [8,9] and *Aspergillus sojae* [10] genomes were sequenced in 2005 and 2011, respectively. Since Inui identified *Aspergillus luchuensis* [11] in black *kōji* (*kuro-kōji* in Japanese) in 1901, many fungal names associated with *kōji* molds have been reported [12,13].

In 2006, the Brewing Society of Japan authorized the certification of "*Aspergillus oryzae*, *Aspergillus sojae* and *Aspergillus luchuensis* are as the national microorganisms (fungi) of Japan" [14].

Enzymology of the National Microorganisms of Japan

α-Amylase, glucoamylase and α-glucosidase of *Aspergillus oryzae*

The invention of "Taka-diastase" by Jōkichi Takamine [15,16] (1854-1922) in 1894 revolutionized the world of industrial enzyme production by fermentation. That same year, Taka-diastase launched the true beginnings of modern microbial enzyme technology, when a rather crude mixture of hydrolytic enzymes was prepared by growing *Aspergillus oryzae* on wheat bran [15,16]. This was then in the West for the time in 1896 [17].

The study of α-amylase (EC 3.2.1.1) from *Aspergillus oryzae* (Taka-amylase A) was developed by Akabori et al. [18]. For additional studies, the Institute for Protein Research in Osaka University was formally founded on April 1st 1958, and Professor Shiro Akabori (1900-1992) was appointed as its first director. Akabori made an important contribution to Japanese enzymology.

It was found, however, that even after repeated crystallization, crystalline Taka-amylase A is still contaminated by traces of proteases that can only be removed by chromatography [19]. Polyacrylamide gel electrophoresis (PAGE) and analytical ion exchange chromatography are used to examine the homogeneity of an amylase preparation. PAGE has shown that crystalline amylase of *Aspergillus oryzae* is homogeneous.

The molecular weight of Taka-amylase A was estimated to be 51,000 ± 500 Dalton (Da) by the combined use of high pressure silica gel (TSK-GEL G3000SW) chromatography and the low angle laser light scattering technique [20]. The amino acid sequence [21] and the genomic nucleotide sequence [22] were determined, for Taka-amylase A.

The Taka-amylase A (TAA) gene [23] was cloned from the genomic library of *Aspergillus oryzae* using synthetic DNA oligomers as probes. The gene was located in a 3.7 Kbp *Escherichia coli* RI fragment. *Aspergillus oryzae* transformants containing an *Eco*RI fragment showed a 2 to 5-fold increase in TAA activity, Complete nucleotide sequence of the gene was determined and found to be 2,040 bp long, with eight introns. The deduced amino acid sequence was compared with that reported by Toda et al. (1982) [21] with the following findings; a presumed signal peptide consisting of twenty-one amino acids was found at the N-terminal, and one insertion, one deletion and ten substitutions of amino acids were observed, which may be due to strain variation. The Taka-amylase A gene (*amy B*) of *Aspergillus oryzae* is induced by starch or maltose.

The molecular structure of Taka-amylase A has been studied at 6Å resolution by X-ray diffraction analysis [24]. The electron density map showed as nm-crystallographic three-fold screw arrangement of the molecules in the crystal. The molecule is an ellipsoid with approximate dimensions of 80 x 45 x 35 Å and contains a hollow which may correspond to the active center. Matsuura et al. (1984) reported that a complete molecular structure model of Taka-amylase A [25] consisting of 478 amino acid residues built with the aid of amino acid sequence data. On the basis of the difference Fourier analysis and the model fitting study, glutamic acid (Glu-230) and aspartic acid (Asp-297), which are located at the bottom of the cleft, were concluded to be the catalytic residues, serving as the general acid and base, respectively.

Aspergillus oryzae produces large quantities of a glucoamylase [26] (α-1,4-glucangluconohydrolase, EC 3.2.1.3) in solid-state cultures ("*kōji*", Figure 1). The nucleotide sequence of an alternative glucoamylase-encoding gene (*gla*B) expressed in solid-state culture of *Aspergillus oryzae* was reported by Hata et al. [27]. The cDNA for *Aspergillus oryzae* glucoamylase was cloned and found to contained an open reading frame encoding 612 amino acid residues [28].

Expression of the glucoamylase [29] (EC 3.2.1.3) encoding gene (*gla*B) which is specifically expressed in solid-state culture, and the tyrosinase-encoding gene (*melO*)), were analyzed using an *E. coli* β-glucuronidase (GUS) reporter assay. These findings suggested that the *melO* promoter would be suitable for hyper-production of heterologous protein in *Aspergillus*. The *gla*B-type glucoamylase selected as the target protein was produced in a submerged culture [29] of *Aspergillus oryzae* under the control of the *melO* promoter. The maximum yield was 0.8 g/l broth, and the total extracellular protein purity was 99%. The results show that the micro-cell of *Aspergillus oryzae* is a precise biochemical factory.

The hydrolysis of glucoside-linkage catalyzed by every carbohydrate-hydrolase is a reaction in which the product retains (α → α or β → β) or inverts (α → β or β → α) the anomeric configuration of the substrate. Glucoamylase and α-glucosidase (EC 3.2.1.20) by releasing α-glucose and β-glucose, respectively, from common substrates containing a α-glucosidic linkage. The distinction between the substrate specificities of the two enzymes was explained by the subsite affinities in their active sites. The amino acid sequences of the regions containing the catalytic sites were compared in α-glucosidases and glucoamylases from various sources. As a consequence, a recommendation was made to group α-glucosidases into two families according to their primary structures. The catalytic reaction mechanisms of carbohydrate-hydrolases were discussed using two significant models of a nucleophilic displacement mechanisms and an oxocarbenium ion intermediate mechanism [30].

Ethyl-α-D-glucoside [31], a trans-glycosylation product of *Aspergillus oryzae α*-glucosidase (EC 3.2.1.20) in Japanese *saké*, is useful for treating skin dermatitis. As fibroblasts of the dermis are involved in skin homeostasis, in the study by Bogaki et al. [32] assessed proliferation, collagen production, and gene expression in normal human dermal fibroblasts (NHDF) cultured in ethyl-α-D-glucoside supplemented medium to investigate the effects of ethyl-α-D-glucoside in NHDF.

Cutinase of *Aspergillus oryzae*

Hydrophobins [33] are amphipathic proteins secreted by filamentous fungi. When *Aspergillus oryzae* is grown in a liquid medium containing the polyethylene succinate coadipate (PBSA), it produces RolA, a hydrophobin, and CutL1, a PBSA-degrading cutinase (EC 3.1.1.74). Secreted RolA attaches to the surface of the PBSA particles and recruits CutL1, which then condenses on the particles and stimulates the hydrolysis of PBSA. Takahashi et al. [33] identified amino acid residues that are required for the hydrophobin RolA-cutinase CutL1 interactions using site-directed mutagenesis and quantitatively analyzed kinetic profiles of these interactions using quartz crystal microbalance (QCM) technique. QCM analyses revealed that Asp-142, Asp-171 and Glu-31, located on the hydrophilic molecular surface of cutinase CutL1, and His-32 and Lys-34, located at the N-terminus of hydrophobin RolA, are critical for the RolA-CutL1 ionic interactions.

RNase T1 and nucleases S1 of *Aspergillus oryzae*

RNaseT1 [34] (guanyloribonuclease, EC 3.1.27.3) was discovered from Taka-diastase Sankyo by Sato and Egami (1957). RNase T1 is a guanosine-specific ribonuclease that cleaves the 3',5'-phosphodiester linkage of single strand RNA. Takahashi [35] revised the primary structure of RNaseT1. Nishikawa et al. [36] corrected that the two histidine residues, His-40 and His-92, but not Glu-58, are indispensable for the catalytic activity of the enzyme. They proposed a revised reaction mechanism in which two histidine residues play a major role, as they do in the case of ribonuclease A (RNase I, pancreatic RNase, EC 3.1.27.5).

During the last decade, protein engineering has been used to identify the residues that contribute to the RNase-T1-catalyzed transesterification [37]. His-40, Glu-58 and His-92 accelerate the associative nucleophilic displacement at the phosphate atom by the entering 2'-oxygen downstream guanosines in a highly cooperative manner. Glu-58, assisted by the protonated His-40 imidazole, abstracts a proton from the 2'-oxygen, while His-92 protonates the leaving group. Tyr-38, Arg-77 and Phe-100 further stabilize the transition state of the reaction.

Ando (1966) discovered *Aspergillus* nuclease S1 [38] (single-strand nucleate endonuclease, EC 3.1.30.1) from Taka-diastase Sankyo. Purification and characterization of single-strand-specific nuclease [39] from *Aspergillus oryzae* were reported by Vogt (1973). The amino acid sequence of nuclease S1 [40] from *Aspergillus oryzae* was determined. Nuclease S1 consists of a single peptide chain of 267 amino acid residues bearing N-glycosylated Asns-92 and -22.

Modification of the carboxylate groups [41] of purified S1 nuclease resulted in a loss of its single-stranded DNAase, RNAase and phosphomonoesterase activities. The inactivation was due to the removal of zinc atoms from the enzyme and this in turn was dependent on the degree of modification. While the removal of one zinc atom resulted in the partial inactivation of the enzyme, removal of the remaining zinc atoms resulted in the complete inactivation of the enzyme. Data obtained with carboxylate-group modification, EDTA-treatment, reconstitution with metal ions, zinc estimation and circular dichroism (CD) analysis of the enzyme suggests that, out of three zinc atoms present in the S1 nuclease, zinc I is easily replaceable and is probably involved in the catalytic activity while zinc II and zinc III are involved in maintaining the enzyme structure.

The single–strand–specific S1 nuclease from *Aspergillus oryzae* is an archetypal enzyme of the S1–P1 family of nucleases with a widespread use for biochemical analyses of nucleic acids. Køval et al. [42] presented the first X–ray structure of this nuclease along with a thorough analysis of the reaction and inhibition mechanisms and of the properties responsible for identification and binding of ligands. Seven structures of S1 nuclease, six of which are complexes with products and inhibitors, and characterization of catalytic properties of a wild type (WT) and mutants reveal unknown attributes of the S1–P1 family. The active site can bind phosphate, nucleosides, and nucleotides in several distinct ways. The nucleoside binding site accepts bases in two binding modes–shallow and deep. S1 nuclease can also undergo remodeling and so adapt to different ligands. An amino acid residue Asp-65 is critical for activity while Asn-154 secures interaction with the sugar moiety, and Lys-68 is involved in interactions with the phosphate and sugar moieties of ligands.

Protyrosinase of *Aspergillus oryzae*

Aspergillus oryzae pro-tyrosinase [43] has a unique feature in that the proenzyme is activated under acidic conditions (pH 3.0). The coding region of the pro-tyrosinase gene, *melO* [44], from *Aspergillus oryzae* occupies 1671 bp of the genomic DNA and is inactive at pH 7.0. Tyrosinase (EC 1.14.18.1; and EC 1.10.3.1) is a type III copper protein and is an important enzyme participating in the process of melanin biosynthesis. For *Aspergillus oryzae* tyrosinase, competitive inhibition was observed with kojic acid (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) [45].

The molecular masses of the pro-tyrosinase and acid-activated tyrosinase were 266 kDa and 165 kDa, respectively, as estimated by gel filtration chromatography. The CD spectra showed that the tertiary and/or quaternary structure changed after acid activation. On the basis of these results, Tatara et al. [46] deduced that the inter-subunit polar interaction is disrupted at pH 3.0, and the tetrameric pro-tyrosinase dissociates to dimers. Tryptophan fluorescence spectra and binding assay of 8-anilino-1-naphthalene sulfonic acid suggested that hydrophobic amino acid residues of the active site were exposed to solvent after acid treatment. It was likely that Cys-108 formed an intermolecular disulfide bond between the subunits of dimeric acid-activated tyrosinase. The dimerization of acid-activated tyrosinase involving the intermolecular disulfide bond is essential for the activity.

Proteolytic enzymes of *Aspergillus oryzae*

In 1950, Crewther and Lennox reported that a strain of *Aspergillus oryzae* grown for ten days at 21ºC, on liquid

medium containing sucrose, tartrate, inorganic salts and ammonium salts as the sole source of nitrogen, was found to produce a highly active crystalline protease system in the culture medium [47].

The characterization of serine proteinase from *Aspergillus oryzae* (oryzin, aspergillopeptidase B, EC 3.4.21.63) was reported by Subramanian et al. and Kalnitsky et al. [48,49].

The cDNA and gene for oryzin have been cloned from several species of *Aspergillus oryzae* [50,51]. The genes from *Aspergillus oryzae* contain three introns, but that from *Aspergillus niger* [52] has only one intron, corresponding to the position of the first intron in the other genes of *Aspergillus oryzae*.

Deuterolysin (EC 3.4.24.39, formerly designated as neutral proteinase II, NpII) from *Aspergillus sojae* and *Aspergillus oryzae*, which contains 1 g atom of zinc/mol of enzyme, is a 177 amino acid single chain protein, with three disulfide bonds and a molecular mass of 19,018 Da [53]. Deuterolysin is extremely stable at 100°C for 10 min, but relatively unstable around 75°C. The other metalloproteinase, neutral proteinase I, has properties similar to those of *Bacillus thermoproteolyticus* thermolysin (EC 3.4.24.27).

By site-directed mutagenesis and the 65Zn-binding assay studies, His-128 and His-132 of deuterolysin from *Aspergillus oryzae* were shown to corresponded to the zinc-binding sequence in thermolysin (EC 3.4.24.27) [54], whereas the third aspartate zinc ligand, Asp-164, of deuterolysin was replaced by Glu-166 in thermolysin. Site-directed mutagenesis experiments showed that mutant D164N had no detectable catalytic activity for mutation from the proenzyme to the mature form or proteolytic activity for salmine hydrolysis [54]. Based on site-directed mutagenesis and zinc-binding sequence experiments, deuterolysin was identified as a member of the Zn2+-metallo-endopeptidase family with a new zinc-binding motif, "ASPZINCIN", defined by the "H128E129XXH132 + D164" motif as aspartic acid (D164) as the third zinc ligand [54].

A new X-prolyl dipeptidyl-aminopeptidase was discovered and purified from *Aspergillus oryzae* grown on solid bran culture medium by Tachi et al. [55]. PAGE showed that the purified enzyme was homogeneous at pH 9.4 and on isoelectric focusing, and its Mr, was 280 kDa by gel filtration. SDS-PAGE, the purified enzyme migrates to a Mr of 145 kDa suggesting that the enzyme was composed of two homogeneous monomers. The isoelectric point pI was 4.2, and the enzyme was inhibited by diisopropylfluorophosphate (DFP), a nerve gas that contributes with the active serine residue. The enzyme has a pH optimum at 7.0-7.5.

L-Glutaminase of *Aspergillus sojae*

L-Glutamate (monosodium glutamate) is the most important taste and flavor component in traditional Japanese soy sauce ("*shoyu*") [4,6]. In soy sauce fermentation, L-glutamate is produced via 2 pathways: [i] direct release from material proteins by proteases and/or peptidases, and [ii] hydrolysis of free-L-glutamate released from material proteins by glutaminases [56] (EC 3.5.1.2). The latter is more important for enhancing taste during soy sauce fermentation because L-glutamine can be converted to pyroglutamate, which has no taste.

Recently, 10 genes encoding glutaminase activity were identified in the *Aspergillus sojae* genome; one of the encoded proteins, GahB, acted as the main glutaminase [57] in soy sauce solid-state culture. Because, the glutaminase activity of the *gahB* disruptant was decreased approximately 90% in *Aspergillus sojae* and *Aspergillus oryzae*, indicating that this enzyme (GahB) accounted for the majority of the glutaminase activity in *Aspergillus* species. As study also found that 4 of the 10 glutaminases, GahA, GahB, GgtA, and Gls, were involved in glutamate production in soy sauce and that peptide-glutaminase [58] reaction of GahA and GahB was very important for enhancing glutamate. *Aspergillus sojae* GahA (AsGahA) was localized toon the cell wall during overexpression in submerged cultures, but AsGahA was purified from the cell surface in submerged culture as a complex of three polypeptides A, B, and C [59].

Proteolytic enzymes of *Aspergillus sojae*

Of the 2847 open-reading frames with Pfam domain scores of 7150 found in *Aspergillus sojae* NRRC 4239 [10], 81.7% had a high degree of similarity to the genes of *Aspergillus oryzae* RIB 40 [8,9]. Comparative analysis identified serine carboxypeptidase (carboxypeptidase C, EC 3.4.16.5) and aspartic proteinases (aspergillopepsin I, EC 3.4.23.18) genes as being unique to *Aspergillus sojae* NBRC 4239. *Aspergillus sojae* NBRC 4239 had 13 serine carboxypeptidases, which was one more than *Aspergillus oryzae* RIB 40, and it had 7 aspartic proteinases (aspergillopepsin I) which was 2 less than *Aspergillus oryzae* RIB 40. Additionally, *Aspergillus oryzae* possessed three copies of the α-amylase (EC 3.2.1.1) gene, while *Aspergillus sojae* NBRC 4239 possessed only a single one copy. In *Aspergillus oryzae*, *amyB* codes for the so-called Taka-amylase A, which is important for amylolysis. Therefore, a decreased copy number of *amyB* orthologues in *Aspergillus sojae* likely accounts for the lower amylolytic ability of *Aspergillus sojae* than that of *Aspergillus oryzae*.

Glucoamylases of *Aspergillus luchuensis*

In 1949, a new γ-amylase [60] from *Aspergillus awamori (Aspergillus luchuensis),* which is now classified as glucoamylase (glucan 1,4-α-glucosidase, EC 3.2.1.3), was found by Kitahara and Kurushima.

Flor and Hayashida [61] reported the production and characteri stics of raw starch-digesting glucoamylase O (GA O) from a pr otease- negative, glycosidase-negative *Aspergillus awamori* var. *kawachi (Aspergillus luchuensis)* mutant. GA O was purified to homogeneity as assessed by SDS-PAGE analysis, and was similar to the parent glucoamylase I (GA I) in the hydrolysis curves toward gelatinized potato starch, raw starch, and glycogen and in its thermostability and pH stability. However, GA O was different in molecular weight and carbohydrate

content (250 kDa and 24.3% for GA O, 90 kDa and ca. 7% for GA I, respectively).

The raw starch affinity site of GA I from *Aspergillus awamori* var. *kawachi (Aspergillus luchuensis)* was determined by Hayashida et al. [62]. The raw starch affinity site was proved to be essential for its absorbability from the active site in the region corresponding to glycol-peptide (Gp-1) liberated from the glucoamylase through the action of subtilisin. Gp-1 consists of 45 amino acid residues, ATGGTTTTATTTGSGGVTSTSKTTTTASKTSTTTSSTSCT TPTAV, hydroxy amino acids being characteristically abundant, and 56 mannose residues. The carbohydrate split GA I could hydrolyze gelatinized substrates almost at the same rate as the native protein, but significantly decreased in the absorbability onto raw starch in comparison with the native protein. Hayashida et al. [63] proposed the "water-cluster-dissociating model" for the hypothetical mechanism of raw-starch-digestion by the absorption of GA I at the affinity site onto starch.

α-1,2-Mannosidase of *Aspergillus saitoi*

An acidic α-1,2-mannnosidase [64] (mannosyl-oligosaccharide 1,2-α-mannosidase, EC 3.2.1.113) has been isolated from *Aspergillus saitoi* R-3813 (ATCC-14332) (now designated as *A. tubingensis* [12,13]). The acid carboxypeptidase (serine carboxypeptidase, EC 3.4.16.5) from *Aspergillus saitoi* contains a large amount of carbohydrates. The new oligosaccharide moieties, Man10GlcNAc2 [65] and Man11GlcNAc2 [66] were identified with α-1,2-mannosidase from *Aspergillus saitoi*. These sugar chain structures have not been found in yeast and animal. In 1998 the proposed structure of the new N-glycan, Man11GlcNAc2, from *Aspergillus saitoi* carboxypeptidase was published [67].

Five crucial carboxyl residues of α-1,2-mannosidase from *Aspergillus saitoi* were identified by site-directed mutagenesis [68]. The molecular properties of recombinant α-1,2-mannosidase overexpressed in *Aspergillus oryzae* were determined [69]. Approximately, 320 mg of pure enzyme was obtained per liter of culture. The recombinant 1,2-α-mannosidase was industrialized by Ōzeki Co. in 1999. Tatara et al. [70] estimated that Glu-124 is a catalytic residue based on the drastic decrease in the $k$cat values of the E124Q and E124D mutant enzymes. Glu-124 may work as an acid catalyst, since the pH dependence of its mutants affected the basic limb. D269E and E411D showed considerable activity. This indicated that the negative charges at these points are essential for the enzymatic activity and that none of these residues can be a base catalyst in the normal sense.

Ca2+, essential for the mammalian and yeast enzymes, but is not required for the enzymatic activity of *Aspergillus saitoi* α-1,2-mannosidase. EDTA competitively inhibits the Ca2+-free α-1,2-mannosidase and does not act as a chelator. The catalytic mechanism of α-1,2-mannosidase may deviate from that of a typical glycosyl hydrolase. This is the first report demonstrating that α-1,2-mannosidase requires no divalent metal cation for the activity. Tatara et al. [71] also reported that a single free cysteine residue (Cys-443) and disulfide bond (Cys-334 and Cys-363) contribute to the thermostability of *Aspergillus saitoi* α-1,2-mannosidase. These results are consistent with the crystallographic data of *Penicillium citrinum* α-1,2-mannosidase in that the corresponding cysteine residues are involved in the formation of disulfide bonds [72].

A yeast mutant capable of producing Man5GlcNAc2 human-compatible sugar chains on glycoprotein was constructed [73]. An expression vector for α-1,2-mannosidase with the "HDEL" endoplasmic reticulum retention/retrieval tag was designed and expressed in *Saccharomyces cerevisiae*. An in vitro α-1,2-mannosidase assay and western blot analysis showed that it was successfully localized in the endoplasmic reticulum. A triple mutant yeast lacking three glycosyltransferase activities was then transformed with an α-1,2-mannosidase expression vector. This is the first report of a recombinant *S. cerevisiae* able to produce Man5GlcNAc2-oligosaccharides [73], the intermediate for hybrid-type and complex-type sugar chain.

Recent years have brought some progress in this area partly because recombinant therapeutic proteins are now very important pharmaceutical agents for treating intractable diseases. Jin et al. [74] first knocked out two genes encoding golgi mannosidases (*MAN1A1* and *MAN1A2*) in HEK293 cells. Consequently, these cells expressed two lysosomal enzymes, α-galactosidase-A and lysosomal acid lipase and glycans on these enzymes were sensitive to endoglycosidase H treatment.

Proteolytic enzymes of *Aspergillus saitoi*

An aspartic proteinase (aspergillopepsin I, EC 3.4.23.18) [75] from *Aspergillus saitoi* R-3813 (ATCC-14332) of black *Aspergillus* ("*kuro-kōji-kin*") activates the generation of trypsin from trypsinogen, which was shown to be associated with the cleavage of the Lys6-Ile7 bond and the release of hexapeptide. Previously, Asp-76 of the aspartic proteinase, aspergillopepsin I, was identified as a binding site of the basic substrate, trypsinogen [76,77].

Consumption of an acid protease derived from *Aspergillus oryzae* was shown to have "bifidogenic effects" [78] in rats, with a considerable increase in *Bifidobacterium* content found in the cecum of rats fed with a high-fat diet supplemented with the *Aspergillus oryzae*-derived Amano protease preparation. The effect cannot be explained by the activity of acid protease at the concentration of 1-g/kg of this preparation, but four-fold higher concentrations of acid protease, compared with those initially used in the diet, were found to cause a significant "bifidogenic effect".

In the human duodenum, trypsinogen is converted to its active form, trypsin (EC 3.4.21.4), by membrane bound entero-peptidase (EC 3.4.21.9). Free trypsin then catalyzes the conversion of additional trypsinogen to trypsin and activates chymotrypsinogen, pro-carboxypeptidases, and pro-elastase. Previously, the acid protease was reported to be released following the trypsinogen activation by the *Aspergillus oryzae*-derived acid-protease [79,80]. It can be assumed that the *Aspergillus oryzae*-derived acid protease-activated trypsin further activates other inactive zymogens in a cascade reaction

and the "bifidogenic effects" of *Aspergillus oryzae*-derived acid protease increase with trypsin activation levels.

A new type of acid carboxypeptidase [81-83] (carboxypeptidase C, serine carboxypeptidase, EC 3.4.16.5) of *Aspergillus saitoi* was identified in 1972. The activity was inhibited by diisopropylfluorophosphate (DFP) at pH 6.0, and was not affected by EDTA at pH 5.2. Takaki et al. [84] reported that lysine, glutamic acid and leucine were released from the anticoagulant decapeptide (SQLQEAPLEK) by *Aspergillus saitoi* acid carboxypeptidase. The acid carboxypeptidase removes acidic, neutral, and basic amino acids as well as proline from the C-terminal position at pH 2~5. The bitterness of peptides from soybean protein hydrolysates was reduced by the acid carboxypeptidase [85]. *Aspergillus saitoi* acid carboxypeptidase gene, *cpdS*, cDNA was cloned and expressed [86]. When *Aspergillus saitoi* acid carboxypeptidase was expressed in yeast cells, acid carboxypeptidase activity was detected in the cell extract and was immune-stained with polyclonal anti-*Aspergillus saitoi* acid carboxypeptidase serum. Site-directed mutagenesis of the *cpdS* indicated that Ser-153, Asp-357 and His-436 were essential for the catalytic activity of the enzyme. It can be concluded that *Aspergillus saitoi* acid carboxypeptidase has a catalytic triad comparing Asp-His-Ser and is a member of carboxypeptidase C (EC 3.4.16.5) family.

Check the Safety

Aspergillus oryzae is generally recognized as a non-phathogenic fungus. A history of safty [87] and non-productivity of aflatoxin is well established for industrial strains, and *Aspergillus oryzae* is considered "general recognized as safe" (GRAS) by the United States Food and Drug Administration (FDA) [88]. Molecular analysis of an inactive aflatoxin biosynthesis gene cluster in *Aspergillus oryzae* was reported by Tominaga et al. [89]. Kato et al. [90] demonstrated that an *Aspergillus oryzae* (NBRC 4177)-specific cyclopiazonic acid (CPA) biosynthetic gene (*cpa*) mediates the conversion of CPA into the less toxic 2-oxocyclopiazonic acid, a new analogue of CPA. The detoxifying properties of *cpaH*, which have been lost in the *Aspergillus flavus* pathway, reflect the relationship of the two species.

Aspergillus sojae is unable to produce aflatoxins and is generally recognized as safe for food fermentation. Chang et al. [91] revealed that the lack of aflatoxin-production in *Aspergillus sojae* results primarily from an early termination point mutation in the pathway-specific *aflR* regulatory gene, which causes the truncation of the transcriptional activation domain of AflR and termination of interactions between AflR and the AflJ co-activator. Both are required for gene expression. In addition, a defect in the polyketide synthesis gene also contributes to its non-aflatoxigenicity.

Yamada et al. [13] reported the draft genome of "*kuro* (black)-*kōji*" mold, *Aspergillus luchuensis* NBRC 4314 (RIB 2604). Key biosynthetic gene clusters of "ochratoxin A" and "fumonisin B" were absent when compared with *Aspergillus niger* genome, showing the safty of *Aspergillus luchuensis* for food and beverage production.

Conclusion

Modern enzymology in Japan has advanced by quantum leaps over the past 100 years. We have seen that the leading studies of enzymology concerning "the national microorganisms of Japan" have long and rich history in biotechnology. As the proverb says that "necessity is the mother of invention". In the review, I conceived the thought that enzymology might be taught on system.

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