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Purification and Characterization of *Bacillus Licheniformis* α-Amylase from Genetically Cloned *E. coli* NM522

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Bacillus licheniformis α-amylase cloned in *E. coli* genetically was purified by ammonium sulfate fractionations, DEAE-Sephacel, Mono-S, and Superose-6 column chromatographies. The highly purified α-amylase preparation showed 221.8 units per mg protein with 30% yield. Disc gel electrophoresis showed one major protein band. The molecular weight of *B. licheniformis* α-amylase produced in *E. coli* was 55,000 daltons by SDS gel electrophoresis. The K_m value of *Bacillus licheniformis* α-amylase produced in *E. coli* was 0.22% and the V_{max} of the enzyme was 0.6-0.7% min by Hofstee plot. The activity of enzyme showed maximum through wide range of pH, from pH 4 to pH 8 but slowly decreased with increasing pH values. The enzyme required Ca^{2+} for its activity. At pH 8.0, the enzyme had about 25% activity after 15 min incubation at 90°C with 1 mM Ca^{2+}.

Introduction

The enzyme α-amylase (α Amy; 1,4-α-D-glucanohydrolase, EC 3.2.1.) is an endoamylase and catalyzes the cleavage of the α-1,4-glucosidic linkage between glucose molecules in starch, glycogen, and dextrins. α-Amylase produces first dextrins, which are subsequently cleaved to maltose, glucose and branched oligosaccharides.

The α-amylases are widely distribured in plant and animal kingdoms and are important for the utilization of polysaccharide *in vivo*. The α-Amylases of different origins exhibit the same specificity, namely catalysis of hydrolysis and have similar enzymatic properties, Ca^{2+} requirements and optimum pHs.[1,2] Interest has been focused on their mode of secretion, regulation of synthesis, protein structure and industrial application. In recent years, the amylase genes of *B. coagulans*,[3] *B. amyloliquefaciens*,[4] *B. licheniformis*,[5] and *B. stearothermophilus*[6,7] have been expressed in either *B. subtilis* and *E. coli*.

Taka-amylase A (TAA), a fungal α-amylase produced by *Aspergillus oryzae* and isolated in high yield,[8] catalyzes the endoamylolytic degradation of starch. The physical and chemical properties of this enzyme (TAA) have been extensively studied,[9] and three-dimensional structure of TAA have been investigated by X-ray structure analysis at 3Å resolution using the multiple isomorphour replacement method.[10] As the complete amino acid sequence determination progressed,[11] molecular model of TAA was completely constructed by fitting skeletal models to the electron density results. The substrate maltotriose-soaked crystal structure of TAA showed a possible binding mode between substrate and enzyme. On the basis of the difference Fourier analysis and the model fitting study, glutamic acid (Glu230) 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.[12-14]

In order to support this acid-base model of TAA as general mechanism of α-amylase, the amino acid sequences inferred from *B. stearothermophilus* and *B. licheniformis* α-amylase gene was compared with amino acid sequence of TAA and the result showed homolozy in three functional groups.[15,16] The *B. licheniformis* α-amylase is the liquefying enzyme most widely used in industry and is the only heat and pH stable α-amylase that is known to show substantial activity in alkaline range at high temperature. This enzyme is stable between pH 6 and pH 11 at 25°C and its optimal temperature is 76°C at pH 9.0. The α-amylase gene of *B. licheniformis* (ATCC 278110) has been cloned[17] and the nucleotide sequence of a DNA fragment of 1,948 base pairs cotaining entire *B. licheniformis* α-amylase was determined completely.[18]

In this study, *B. licheniformis* α-amylase was produced in *E. coli* and was purified to homogeneity. The kinetic parameters, pH-optimums and thermostabilities of the α-amylase were determined.

Materials and Methods

Materials

Protein marker was purchased from Dalton. DEAE-Sephacel, Mono-S, Superose-6 were purchased from Pharmacia-

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LKB. Soluble starch, lysozyme, pronase, RNAse A, spermidine, D-cycloserine, ampiciline and Maltoheptoase were purchased from Sigma. EcoRI, PstI and T4-DNA ligase were purchased from KOSCO. Beef extract and peptone were purchaed from Difco. Ammonium sulfate, DNS(2-OH-3,5-dinitrobenzoic acid) and Rochell salt (sodium potassium tartarate) were of the highest purity available.

Methods

Strain and culture condition for purifiction. Each of E. coli NM522, which had B. licheniformis α-amylase was grown in 3 liters of the LB medium containing 1% soluble starch and 35 μg/ml of ampiciline at 37℃ overnight in fermentor to the stationary phase. Then the cells were harvested with Sorvall GSA rotor at 7,000 rpm for 5 min.

Cloning of α-amylase gene from B. licheniformis. Isolation of chrosomal DNA from B. licheniformis was done as described in Palva.[19] Standard DNA manipulation were performed as described in Maniatis et al.[20] Transformation of E. coli MC1061 was performed by the method of Mandel[21] with some modification.[20] The resulting transformed cells were plated on LB plates containing 12.5 μg/ml tetracycline-1% starch and were grown at 37℃ for 12 hours.

The screening of α-amylase gene bearing colonies was performed as suggested by Mielenz[6] with some modifications. The 3 mg/5 ml of D-cycloserine was overlayed onto the incubated cells of tetracycline-starch-LB plate and the cells were further incubated at 37℃ for 5 hours. Then the resulting plates were tested by pouring 1% I₂/10% KI on the surface of the plates. Transformants were screened for the proper insert by mini-preparation of their plasmid DNA.[22]

Assays of α-amylase.

a. DNA method

The modified method of Bernfelt[23] was used to determine the quantities of α,β-glucose liberated after enzyme-substrate reaction.

To determine the amylase activities, 500 μl of soluble starch solution, 400 μl of buffer solution and 100 μl of enzyme solution were mixed well and incubated at 37℃. After reaction for 10 min, the enzyme solution 100 μl was mixed with 300 μl of the DNS reagent and then the mixture was colored in boiling water for 10 min and then the mixture was cooled in tap water and 2.1 ml water was added. The O.D. of the solution was measured at 525 nm.

The standard curve of maltoheptoase was used for measuring the enzyme activities. One unit is defined as the amount of enzyme producing 1 mg of maltoheptoase with 1 ml of enzyme and substrate solution under the conditions described above.

b. Iodine method

The starch polymers form a complex with iodine and the absorbance at 700 nm was detected to determine the remained starch. Amylase activity was measured by assaying of dextrinizing power, which was proposed by Fuwa[24] and modified by Takahashi.[25]

The dextrinizing power was calculated by the equation of $D.P. = (D_0 - D)/D_0 \times 100/10$, where D is optical density of the reaction mixture, D_0 is optical density of the control. Consequently, one dextrinizing power unit (D.P.U) of α-amylase was defiened as the amount of enzyme causing 10% reduction in the intensity of blue color of starch-iodine under the standard assay condition.

Estimation of protein. The protein was estimated according to the method of Bradford[26] with slight modification.[27] The crystalline bovine serum albumin was used as standard. To estimate the concentration of protein, 10 mg/ml-1 mg/ml, 1 mg/ml-0.1 mg/ml, 0.1 mg/ml-0.01 mg/ml of bovine serum albumin standard curves were used.

The proteins of eluted column fractions were estimated by measuring the optical densities at 280 nm.

Purification of B. licheniformis α-amylase from cloned E. coli cells. All the procedures for the purification of α-amylase was performed at 4℃ unless otherwise mentioned.

Making crude extract.

The harvested cells were suspended in 0.05 M Tris-malate NaOH (pH 7.0) buffer, then the suspension was sonicated for about 20 min (Heat-system ultrasonic, Inc., Model W-225 R). Then the unsonicated cells and cell debris were removed by centrifugation at 14,000 g for 10 min with Sorvall SS-34 rotor. To the solution, the solid ammonium sulfate was added with gentle stirring to 40% saturation and centrifuged at 14,000 g for 10 min with Sorvall SS-34 rotor. More ammonium sulfate was added to 80% saturation and to stand overnight at 4℃. Then the precipitation was dissolved in 10 ml of 0.05 M Tris-malate NaOH (pH 7.0) buffer. To remove the ammonium sulfate, the solution was dialyzed against the same buffer.

a. DEAE-Sephacel column chromatography

The enzyme solutions were applied to a column of DEAE-Sephacel (3.8 cm×4 cm) which had been equilibrated with 0.05 M Tris acetate (pH 7.5) buffer. A further 100 ml of the same buffer to pass through the column with the flow rate of 90 ml/hr, and 2 ml fractions were collected. The effluent was concentrated with ultrafiltration to about 1/20 volume and dialyzed against 0.01 M sodium phosphate buffer (pH 6.0).

b. Mono-S column chromatography

The dialyzed sample was applied to a FPLC column of Mono-S in 0.01 M Sodium phosphate buffer (pH 6.4). The column was washed with the same buffer, and a linear gradient elution was carried out with 10 ml of each of 0 and 0.7 N NaCl in the same buffer. The flow rate was adjusted to 30 ml/hr and 0.5 ml fractions was collected. The active fractions were pooled and concentrated by ultrafiltration to 430 μl and dialyzed against 0.01 M sodium phosphate buffer (pH 6.0).

c. Superose 6 column chromatography

The dialyzed sample was applied to a FPLC column of gel filtration (Superose 6) in 0.01 M sodium phosphate buffer (pH 6.0). By passing about the same column volumes of buffers at the flow rate of 3 ml/hr, the 0.3 ml fractions were collected. Then the active fractions were pooled and concentrated by ultrafiltration.

SDS-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS) was performed in 10% (w/v) slab gels. Gels were stained for protein bands with Coomassie brillant blue. Protein markers were used as follows; Phosphorylase b (M.W. 97,400), Bovine serum albumin (M.W. 66,000), Albumin, egg (M.W. 45,000) and Trypsinogen (M.W. 24,000).

Determination of kinetic parameters. The kinetic parameters, K_m, and V_{max}, were determined from the Hofstee

plots with soluble starch as substrate.

In order to determine the linear range of the enzyme-substrate reactions, two control reactions were performed. The first is to determine the effect of enzyme concentration and reaction times on the formation of product. 1% soluble starch in 500 μl of 0.1 M Tris-HCl (pH 8.0) and 100 μl of 1 unit/ml enzyme solution, 2 unit/ml enzyme solution, 3 unit/ml enzyme solution were incubated at 37℃ for 5 min, 10 min, 15 min, 20 min, then the maltoheptoase liberated was measured by the DNS assay method.

The second is to determine the effect of substrate concentrations on the enzyme activity. 500 μl of each 0.05%, 0.1%, 0.2%, 0.5%, 1.0% starch solutions in 0.1 M Tris-HCl (pH 8.0) and 100 μl of enzyme solution of which their unit which was determined in the first control reactions was incubated at 37℃ for a constant time which were determined in the first control reaction. Then the maltoheptoase liberated was determined by the DNS assay method.

Determination of pH optimum. In order to determine the pH-optimum, Britton-Robinson buffer was used for pH 3, 4, 5, 6, 7, 8, 9, 10. 500 μl of 1% soluble starch in water, 200 μl of 2x pH buffer, 200 μl of water, and 100 μl of enzyme diluted with water were incubated for 10 min at 37℃, then the activities were measured by DNS assay method.

Determination of thermostability. Enzyme solutions were incubated at 40, 50, 60, 70, 80, and 90℃ water bath respectively for 15 min, then the remaining activities were measured at 37℃ for 10 min incubation with 100 μl of enzyme solutions, 400 μl of buffer, and 500 μl of 1% starch solutions. Then the activities were measured by the DNS assay method. This reaction was performed at pH 8 with and without 1 mM Ca^{2+}.

Results and Discussion

Recently thermostable α-amylases of Bacillus species have become important for the stability related structural studies. To investigate the structural relationships with thermostability and for protein engineering studies, α-amylase genes of cloned Bacillus species were used as a means of for more easily production of enzymes and for site-directed mutagenesis.

Several α-amylase coding genes of Bacillus species have been up to now cloned in *E. coli* such as the α-amylase genes of *B. subtilis*, such as the α-amylase genes of *B. amyloliquefaciens*.[19] The cloning and expression of *B. licheniformis* α-amylase gene into *E. coli* performed in this studies was well reproduced as published by Kim and Yang.[17] The size of *B. licheniformis* chrosomal DNA showing α-amylase activities was inserted into PBR322 cloning vector. The 1.5 Kb α-amylae coding region together with vector are shown in Figure 1. The fact that α-amylase is also secreted from *E. coli* makes the screening more easier (Figure 2), because there is no need for D-cycloserine treatment. It was also observed that the wheat α-amylase signal peptide is recognized in *E. coli* resulting in secretion of the enzyme into the periplasmic space by Gatenby *et al.*[28]

Mass production of *B. licheniformis* α-amylase is possible with *E. coli* cells contained α-amylase gene. Overexpression of the enzyme is in progress in *E. coli* but not established
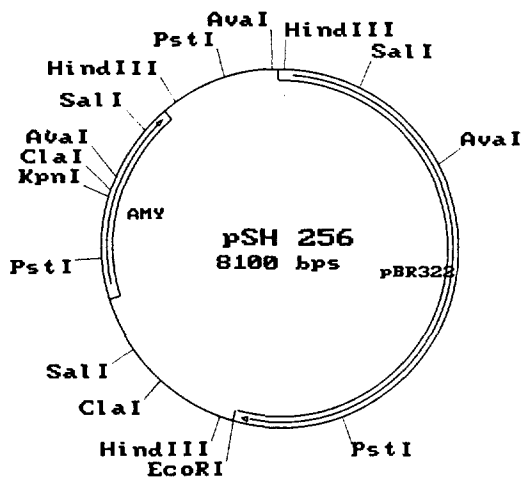
Figure 1. Restriction Map of α-amylase gene bearing plasmid, pSH256. The 4.3 Kb of right hand side represent the pBR322 and about 3.8 Kb of left hand side represent the insert. The region "AMY" represents about 1.5 Kb amylase coding region.
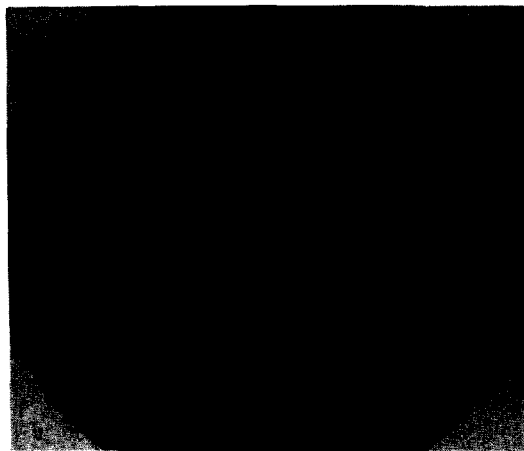

Figure 2. Screening of colonies bearing *B. licheniformis* α-amylase gene. The *E. coli* MC1061 with cloned α-amylase gene were picked up onto the LB-ampicillin starch plate. The colonies were picked and 3 mg/5 ml D-cycloserine was overlayed onto the plate after 5 hours growth. Then after further growth, the amylase activity was tested by pouring 1% I_2/10% KI on the surface of the plate. The colonies with clear spot are the *E. coli* colonies with the cloned amylase gene.

the method for solubilizing the enzyme in cluster.[17]

In this studies, FPLC system was adopted for the first time in order to get highly purified α-amylase with more faster and greater yield. Overall purification was 78 fold with 30% recovery of the enzyme. The entire purification scheme is summerized in Table 1. By fractionation with ammonium sulfate 40-80%, most of α-amylase activities was recovered. Since the enzyme does not bind to DEAE-Sephacel, enzyme was eluted in the void volume. However, this step is necessary for removing contaminant protein to go to the next cationic exchange chromatography step. The elution profile of Mono-S FPLC column indicated that α-amylase active fractions were eluted between 0.3-0.4 M NaCl gradient as shown

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Table 1. Purification table of α-amylase

Purification Steps	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
(NH₄)₂SO₄ Fractionation	220.5	705.6	3.2	100
DEAE-Sephace column	51.5	638.6	12.4	90.5
Mono-S Column	4.1	410.9	100.2	58.2
Superose-6 column	0.85	221.8	249.2	30.0
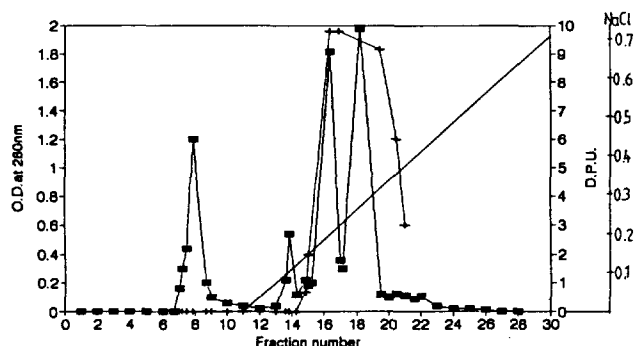
Figure 3. Mono-S column elution profile of *B. licheniformis* α-amylase by FPLC. The Mono-S column was equilibrated with 0.01 M Na-Phosphate (pH 6.4). The linear gradient elution was applied to the column with 10 m*l* of each 0.0 and 0.7 N NaCl in the above buffer. The flow rate was adjusted to 30 m*l*/hr and 0.5 m*l* fractions were collected.
(■) O.D. at 280 nm, (+) D.P.U.
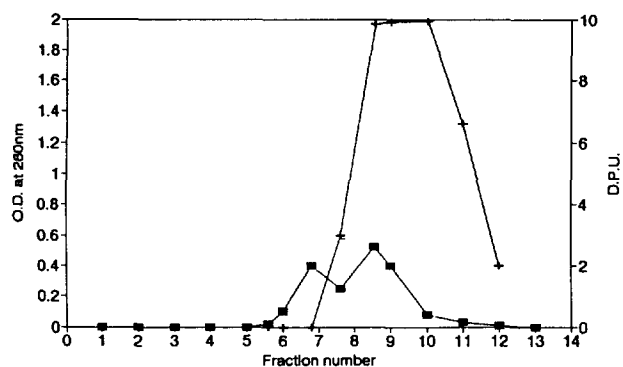
Figure 4. Superose 6 column elution profile of α-amylase by FPLC. The FPLC gel filtration column was passed with 0.01 M Sodium phosphate buffer (pH 6.0). The flow rate was 3 m*l*/hr and 0.3 m*l* fractions were collected.
(■) O.D. at 280 nm, (+) D.P.U.

in Figure 3. The last peak eluted after NaCl gradient had the most of the α-amylase activity actually, however second and third peaks looks to have enzyme activity in the chromatogram because of active enzyme fraction eluted with certain time intervals. Further purification was achieved by Superose 6 gel filtration step showing the elution profile in Figure 4.

After the last gel filtration of Superose 6 step, the purified α-amylase was tested its purity by applying polyacrylamide gel electrophoresis. The SDS-PAGE of the purified α-amylase
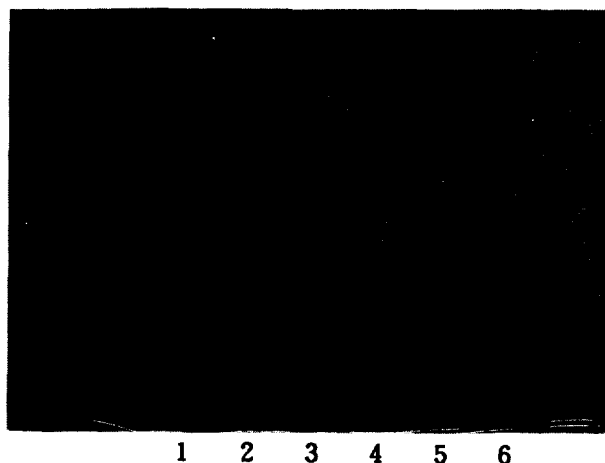

Figure 5. SDS-PAGE analysis of α-amylase active fractions obtained after each steps of purification.
Lane 1 : Marker protein (From; Phosphorylase a (M.W. 97,400), Bovine serum albumin (M.W. 66,000), Albumin, egg (M.W. 45, 000), Trypsinogen (M.W. 24,000); Lane 2 : Fraction after ammonium sulfate fractionation; Lane 3 : α-amylase active fractions from DEAE-Sephacel; Lane 4 : α-amylase active fractions from Mono-S column; Lant 5 : α-amylase active fractions from Superose-6 column; Lane 6 : Marker proteins.
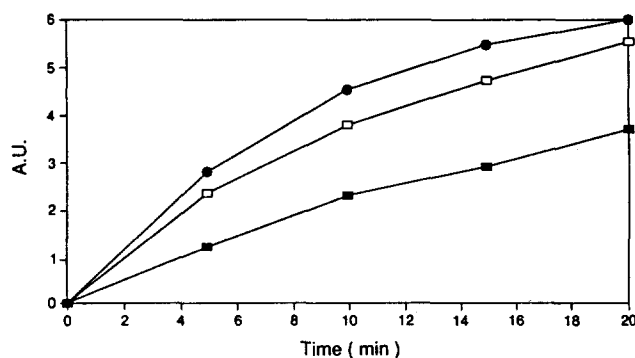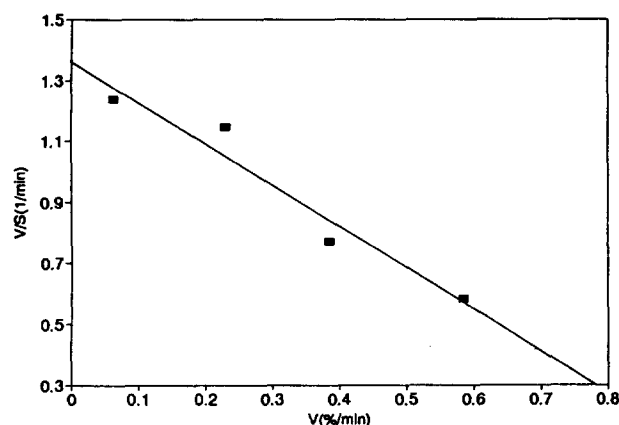

Figure 6. Effect of enzyme concentration with different reaction on the formation of product. The reaction mixture was 1% soluble starch in 0.1 M Tris-HCl (pH 8.0) 50 m*l* and (■) 1 unit/m*l* enzyme, (□) 2 units/m*l* enzyme solution 100 μ*l*, (○) 3 units/m*l* of enzyme were incubated at 37°C for 5 min, 10 min, 15 min, 20 min. Liberated maltoheptoase was measured by DNS assay method.

showed one major protein band and molecular weight was estimated to be 55,000 daltons by comparing with the marker proteins as indicated in the method (Figure 5). It is reported that the α-amylae isolated from *B. licheniformis* showed four active protein bands.[29] The molecular weight of α-amylase calculated from DNA sequence is well agreed.[17] The molecular weight of *B. licheniformis* α-amylase was reported to be 22,500.[29] It indicated that α-amylase produced in *E. coli* exists as dimeric form. The α-amylase consisted of two polypeptide chains linked by two disulfide bridges.[30]

Effect of the reaction time and enzyme concentration was carried out for kinetic studies. The activity unit versus reaction time is linearized for 10 minutes of 1 unit/m*l* enzyme.
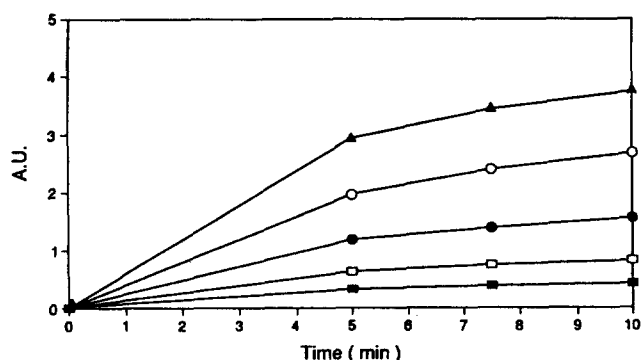
Figure 7. Effect of substrate concentration on α-amylase. 500 ml of each 0.05%, 0.1%, 0.1%, 0.2%, 0.5%, 1.0% starch solutions in 0.1 M Tris-HCl (pH 8.0) and 100 μl of enzyme solution of which their unit determined in the Figure 6 was incubated at 37℃ for constant time determined in Figure 6 control reaction. The maltoheptoase liberated was determined by the DNS assay method. (■) 0.05%, (□) 0.1%, (●) 0.2%, (○) 0.5%, (▶) 1.0%.


Figure 8. Hofstee plot of α-amylase. Soluble starch as substrate was used to determine the V_{max} and K_m.

The results are shown in Figure 6. The enzyme-substrate reactions gave linear relationship.

The effect of substrate concentration on the enzyme activity was tested to establish optimum assay method. The activity unit *vs.* reaction time was linearized for 5 minutes for α-amylase. The results are shown in Figure 7. On the basis of the above results, the kinetic parameters, K_m and V_{max}, were determined from Hofstee plot (Figure 8). Since soluble starch with average molecular weight was used as substrate, kinetic parameters were expressed in percentage. The K_m and V_{max} values were estimated to be 0.22% and 0.60%/min respectively.

The amylases of Bacillus species are generally stable in the pH range from 5.5 to 8.0 and optimal pH ranges are from 4.8 to 6.5.[31] The optimum activity profiles of α-amylase are shown in Figure 9.

The activity profiles of the enzymes (without Ca^{2+}) at different temperatures at pH 8.0 are shown in Figure 10. And the activity profiles of the enzyme with 1 mM Ca^{2+} at different temperature, at pH 8.0 are shown in Figure 11. It is reported that α-amylase isolated from *B. licheniformis* showed optimum activity at higher than 60℃.[32,33]
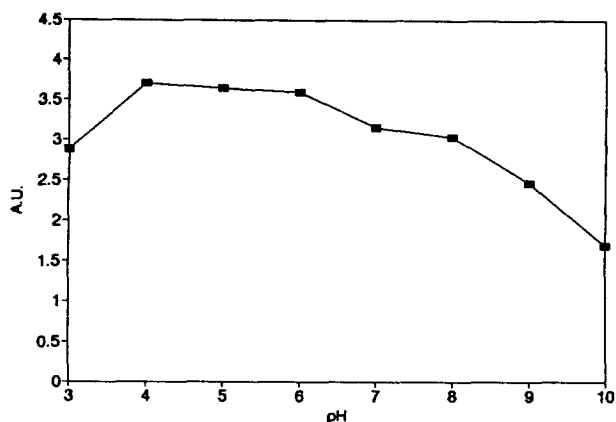
Figure 9. Profiles of pH optimum. Britton-Robinson buffer was used for pH 3, 4, 5, 6, 7, 8, 9, 10. 500 μl of 1% soluble starch in water, 200 μl of 2x pH buffer, 200 μl of water and 100 μl of enzyme diluted with water were incubated for 10 min at 37℃.
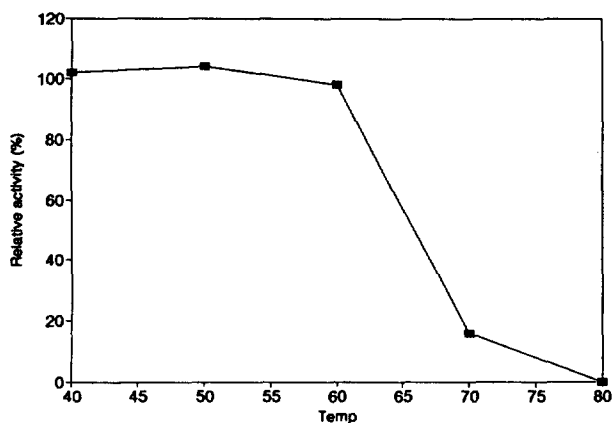
Figure 10. Test of thermostability of α-amylase. The activity profiles of α-amylase (without Ca^{2+}) at 40, 50, 60, 70, 80, 90℃, at pH 8.0 were tested.
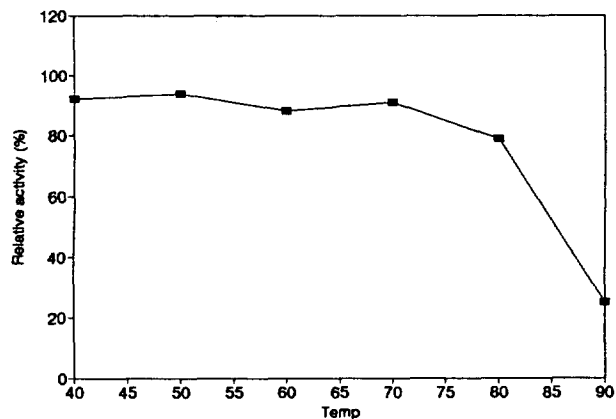
Figure 11. Thermostability test of α-amylase with 1 mM $CaCl_2$ at pH 8.0.

The α-amylases are known for calcium metalloenzymes with at least one calcium atom per one enzyme molecule and in the presence of this cation, they are extremely stable toward temperature, pH, denaturating reagents such as urea

and proteases.[34] In the Ca^{2+} added enzyme reactions, the enzyme was much more stable after 80℃ incubation than the reaction mixture without Ca^{2+}, and after 90℃ incubation α-amylase had 25% activities as previously reported.[29] Therefore, it can be shown that Ca^{2+} acts to stabilize a tertiary structure of the enzyme which yields catalytically active molecule as indicated earlier.[34] The enzyme containing a −SH group at the active site are also reported to be thermostable[35] and high proline content of the enzymes were correlated with a low degree of α-helical structure; the molecules was postulated as the reason for the observed marked thermostability of enzyme.

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