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Saccharopine, a New Amino Acid in Baker's and Brewer's Yeast

I. Isolation and Properties *

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A new ninhydrin-positive substance, discovered by paper chromatography, has been isolated from hot water extracts of fresh baker's and brewer's yeast. By means of strongly acid and weakly basic ion exchange resins the amino acids of such extracts have been divided into one fraction containing the neutral and basic amino acids, and another containing the acid amino acids including the new compound, for which the name *saccharopine* is proposed.

Addition of ethanol to the eluate (pH 2) containing the acid amino acids, followed by recrystallization of the resulting precipitate from water (pH 3), affords pure saccharopine in amounts of 0.3 – 0.5 g from 2 kg batches of yeast. An anhydrous, analytical specimen possesses the composition $C_{11}H_{20}O_6N_2$ and contains three acid and two basic functions, the latter attributable to one primary and one secondary amino grouping.

On treatment at 125° with strong acid or base, saccharopine is partly transformed into a new compound, probably a lactam resulting from cyclodehydration involving the secondary amino grouping.

In the course of transamination studies in this laboratory, a ninhydrin-positive spot, which could not be assigned to any previously known amino acid, was observed on paper chromatograms of hot water extracts of baker's yeast (Fig. 1). The same amino acid pattern was found when extracts of mechanically disintegrated or freeze-dried yeast were utilized.

The unknown constituent may be identical with a component of dried baker's yeast, described by Lindan and Work [1] as 'No. 15', and with a substance in *Torulopsis utilis* indexed by Miettinen [2] as 'No. 30'. These authors reported

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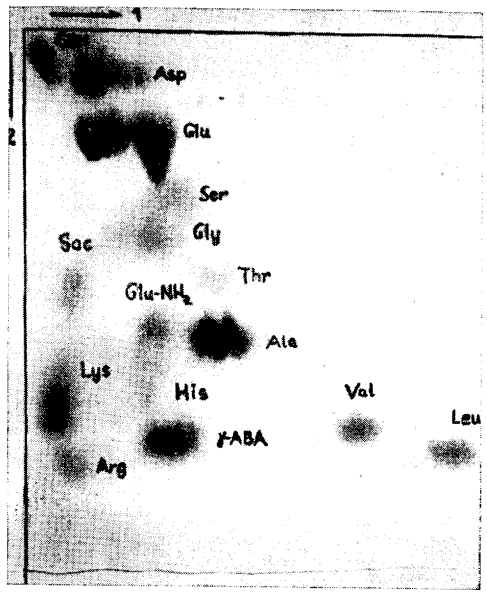

Fig. 1. Two-dimensional chromatogram of a hot water extract of baker's yeast after ion exchange on an acid resin and elution with ammonia. The applied volume is 5 μl, corresponding to 50 mg of fresh yeast. Solvent 1: butanol-pyridine-water (1:1:1). Solvent 2: phenol-water (3:1) with added NH_3 and cyanide. Spray-reagent: 0.2 % ninhydrin in acetone. Sac = saccharopine; other abbreviations are those commonly used. Glutamic acid appears as a double spot.

a remarkable acid stability of paperchromatographic isolates of the above-mentioned unknown compounds, though the treatment in both cases was accompanied by the production of minor amounts of a considerable number of the ordinary amino acids. Similarly, it was observed in this laboratory that a spot eluate of the unknown yeast constituent on acid hydrolysis gave mainly unchanged starting material, yet accompanied by a derivative thereof (*cf.* also Ref.[2]) besides small amounts of a number of the common amino acids. The presence in yeast extracts of a peptide, indistinguishable by paper chromatography from the unknown constituent, is consonant with the observed patterns. The present paper reports the isolation of the acid-stable compound, identified as an amino acid, for which the name *saccharopine* is proposed *.

Isolation experiments were initially performed on hot water extracts of baker's yeast. From such extracts the total amino acid fraction was absorbed on a strongly acid ion exchange resin. On standing, the acid effluent deposited

* This name is coined in accord with the biological origin of the amino acid and is furthermore in conformity with names such as octopine and lysopine, two naturally occurring amino acids with which the present compound is structurally related as demonstrated in a subsequent communication defining also the stereochemical specification [3].

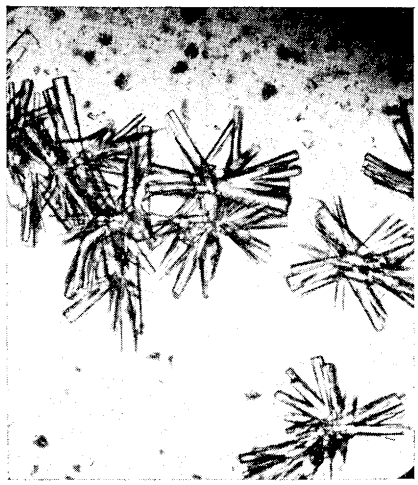

Fig. 2. Saccharopine from baker's yeast, recrystallized from an aqueous solution. 120 × natural size.

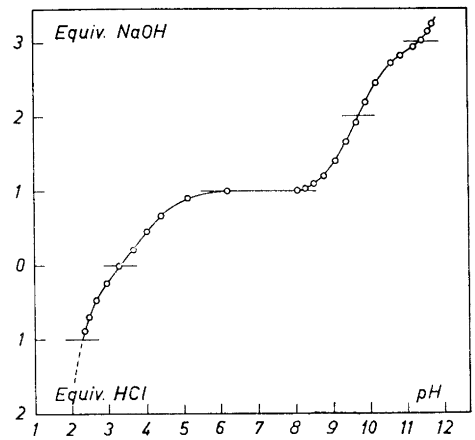

Fig. 3. Titration curve for saccharopine in water. Conditions are those outlined in the experimental part.

a precipitate which on basis of ultraviolet absorption data and its behaviour on acid hydrolysis appeared to be a nucleoprotein. Glutathione was the only detectable ninhydrin-positive substance in the effluent. The amino acids were displaced with ammonia, and the eluate subsequently passed through a weakly basic anion exchange resin in its acetate form, retaining all acid amino acids including saccharopine. The latter were then displaced from the column by hydrochloric acid, and the residue from the eluate was purified by repeated reprecipitations from aqueous solution at pH 2.0 with ethanol. Finally, adjusting of an ammonia solution to pH 3.5 caused saccharopine to separate in yields varying from 0.3 to 0.5 g per 2 kg of fresh yeast. Repetition of this purification procedure afforded a beautifully crystalline specimen of pure saccharopine (Fig. 2). When applied to extracts of brewer's yeast the above procedure yielded a crystalline compound which on basis of chromatographic properties and crystalline appearance proved identical with saccharopine from baker's yeast. Furthermore, no melting point depression was observed on admixture of the two preparations.

Direct isolation of saccharopine from the ammonia eluate of the acid ion exchange column was attempted by adjustment of the pH value to 2.00. However, addition of ethanol and reprecipitation with the same solvent resulted in a considerably smaller yield of a product, heavily contaminated with glutamic acid and lysine, both of which were difficult to remove by recrystallizations.

The new amino acid is sparingly soluble in water and ethanol but readily soluble in bases and strong acids. It gives off water over phosphorus pentoxide at 100°, and analysis of the hygroscopic, anhydrous preparation indicated its

elemental composition to be $C_{11}H_{20}O_6N_2$. Electrometric titration in water and titration in acetone revealed the presence of three acid and two basic functions in the amino acid. From the curve presented in Fig. 3 the following apparent dissociation constants were determined: pK_2 2.6, pK_3 4.1, pK_4 9.2 and pK_5 10.3.

The ninhydrin colour yield was determined according to Moore and Stein [4]. When calculated on molar basis, the colour yield was 90 %, relative to leucine. With acetaldehyde and sodium nitroprusside in alkaline solution, saccharopine produced an intensely blue colour, indicative of secondary amines [5]. Hence, the amino acid contains a primary as well as a secondary amino grouping.

On heating to 125° in 6 M hydrochloric acid, saccharopine was slowly but exclusively converted into a new compound. Even after a heating period of 16 days, however, the conversion was not complete. Similar treatment with saturated barium hydroxide likewise resulted in partial transformation of saccharopine into a compound, which is chromatographically indistinguishable from the product obtained on acid treatment. In accord with the strongly acid character of the new compound (*vide infra*), the paperchromatographically determined R_F-values are highly dependent on the pH-value of the applied solution in solvent systems devoid of buffering capacity towards acids (*e.g.* 0.26 in strongly acid solution, 0.15 in neutral or basic solution, in the solvent system *n*-butanol: acetic acid:water (4:1:5)). As expected, such variations are not encountered when phenol:water:ammonia or butanol:pyridine:water are used as solvent systems.

The first isolate of the transformation product was obtained from a one-dimensional chromatogram developed in butanol:acetic acid:water. Upon electrophoresis, the compound migrates towards the anode, both in citrate buffer (pH 2.5) and 0.1 M acetic acid (pH 3.3), whereas saccharopine travels towards the cathode. Consequently, the conversion product is a stronger acid than saccharopine. In 1 M acetic acid (pH 2.2) both substances migrate towards the cathode. By means of continuous paper electrophoresis at pH 3.8 about 15 mg of the transformation product, contaminated with ammonium chloride, was isolated from acid-treated, partially converted saccharopine. Upon electrometric titration with 1 M sodium hydroxide, a pK_2-value of 3.2 was determined for the conversion product, which proved to contain two acid and one basic groupings. The minimum molecular weights 268 and 258 were determined by electrometric titration and micro-Kjeldahl analysis, respectively, on the assumption that two nitrogen atoms are present. The latter molecular weight, which is considered to be the most reliable, indicates loss of the elements of water during the acid-induced transformation of saccharopine. The protolytic properties, together with the fact that acid-treated solutions of saccharopine give unchanged colour yield with ninhydrin, suggest that intramolecular cyclodehydration between a carboxylic group and the secondary amine function takes place, as further discussed in the subsequent communication [3].

Preliminary experiments on the influence of saccharopine on the growth of various strains of *B. subtilis* on agar showed a marked inhibitory action on four strains in concentrations as low as 10^{-5} M. In two cases, a growth-promoting effect of saccharopine was observed. No effects were observed, however, when liquid media were used.

EXPERIMENTAL

Melting points are determined in capillary tubes with the thermometer (Anschütz) fully immersed in the oil bath.

Ion exchange columns. The resin Zeo-Karb 225 (300 g) in H^+-form was packed wet into a glass tube with an internal diameter of 3.4 cm (the height of the resin bed was about 37 cm). The flow rate of the solutions was kept between 0.6 and 0.8 ml cm^{-2} min^{-1}. A column of the resin Amberlite IR-4B (analytical grade) in its acetate form was prepared as follows: a glass tube with an internal diameter of 3.7 cm was packed wet with 150 g of Amberlite IR-4B (OH^-). The resin was converted into its Cl^--form by passing 450 ml of 3 M HCl, followed by 3 l of water, through the column, which was then reconverted into its OH^--form with 1 200 ml of 1 M NH_3 followed by 1 500 ml of water. Finally, the resin was transformed into its acetate form by allowing 1 500 ml of 1 M acetic acid to pass through the column, followed by 300 ml of CO_2-free water. During all operations, the flow rates were not allowed to exceed 0.3 ml cm^{-2} min^{-1}.

Isolation of saccharopine from baker's yeast. Fresh baker's yeast obtained from *A/S Dansk Gæringsindustri* was used. The yeast was stored at + 4°, but never for more than a fortnight.

A suspension of 2 kg of yeast in 2 l of water was prepared and poured into 8 l of boiling water under constant stirring. The mixture was boiled for 60 min and then set aside at room temperature overnight. The sediment was filtered off on Büchner funnels with Hyflo-supercel as a filter aid. The filtrate was evaporated *in vacuo* to about 150 ml (with tributyl phosphate as an anti-foam agent), and the concentrate together with the rinsing water (150 ml) was centrifuged at 15 000 × *g* and 20° for 60 min. The supernatant was then diluted to 350 ml and passed through the cation exchange column, followed by 2 l of water. The first 100 ml of the effluent was transparent (pH 6). Then pH decreased to about 1, and the next 500 ml-fraction was turbid and of a greyish-brown colour. The pH-value now rose again to between 2 and 3, and the eluate became less cloudy. The last part of the yellowish effluent was perfectly clear (pH 5—6). All effluent fractions were discarded.

The entire amino acid fraction was now eluted with 2 l of 1 M ammonia, the first half of the eluate being slightly cloudy (pH 3—4). The pH-level then increased to 10, and the eluate became transparent and yellow. The solution was evaporated to dryness *in vacuo*, and the residue was redissolved in 100 ml of water. This solution was then passed through the anion exchange resin, and the column was washed with 500 ml of CO_2-free water. The first 100 ml was yellow (pH 6), while the remainder was colourless, with a final pH-value of about 3. This effluent, containing only neutral and basic amino acids, was discarded.

The acidic amino acids, including saccharopine, were now eluted with 800 ml of 3 M HCl, passed through from the bottom of the column. The first 600 ml of eluate were almost colourless (pH 6). Then the pH-level fell to below 1, and at the same time the solution acquired an intensely yellow colour. The eluate was evaporated to dryness *in vacuo* on a water bath kept at 40—45°. Excess HCl was removed by evaporating the residue twice with 100 ml-portions of water *in vacuo*, and the solid was dried over NaOH *in vacuo* at 40° for 6 h. It was then redissolved in 50 ml of water, the solution was treated with 5 g of acid-washed, activated charcoal (B.D.H.) and filtered. The combined filtrate and washing liquid was diluted to a volume of 65 ml. This solution, which was clear and faintly yellow, contained only saccharopine, glutamic acid, aspartic acid, and glutathione, as apparent from two-dimensional chromatograms.

The first ethanol precipitate was obtained by adjusting the concentrated eluate from the exchange column to pH 2.00 with conc. ammonia and then adding 8 volumes of 96 % ethanol. A crystalline precipitate began to appear after a few minutes. The suspension was kept at + 4° for 72 h, the crystals were collected and washed, first with ethanol-HCl (8 volumes of 96 % ethanol and 1 volume of 0.01 M HCl) and then with acetone. After drying over silica gel and potassium hydroxide *in vacuo*, the yield was 0.8—1.5 g. The product consisted mainly of saccharopine, contaminated with glutamic acid and traces of aspartic acid and glutathione. The solid was dissolved in 40 ml of water, the solution was clarified by centrifugation, pH was adjusted to 2.00 by addition of 3 M HCl, and 8 volumes of 96 % ethanol were added. After a few minutes, a crystalline sediment began to appear which was collected after 72 h at 4°, washed with ethanolic

HCl and acetone, and finally dried over silica gel and potassium hydroxide *in vacuo* (0.4—0.6 g). Paper chromatography of this product revealed a slight contamination with glutamic acid, aspartic acid and glutathione.

The partly purified material was redissolved in 5 ml of 1 M ammonia and pH was adjusted to 3.5 with 3 M HCl. After a few minutes, saccharopine crystallized in fine needles. After standing at 4° for 72 h, the crystals were filtered off and washed, first with ethanol-HCl and then with acetone. After drying over silica gel and potassium hydroxide *in vacuo*, the yield was 0.3—0.5 g. The product was still faintly yellow, hygroscopic and contaminated with traces of glutamic acid. After a new treatment with charcoal and reprecipitation, 1 g of this product yielded 700 mg of a colourless, homogeneous specimen, which was dried over phosphorus pentoxide at 100° and 0.1 mm for 4 h before analysis, m.p. 257—259° (decomp.). (Found: C 47.9; H 7.41; N 10.12 (Dumas). Calculated for $C_{11}H_{20}O_6N_2$: C 47.81; H 7.30; N 10.14), $[\alpha]_D^{23}$ + 33.6° (*c* 1.0, 0.5 N HCl); + 8.1° (*c* 2.2, 0.5 N NaOH).

Isolation of saccharopine from brewer's yeast. Following the above procedure the same amino acid was isolated from brewer's yeast grown on hop-free wort.

Electrometric titration of saccharopine. The titration curve in the pH-range below 7 (Fig. 3) was determined by titration with 0.5 M sodium hydroxide of 33.5 mg of saccharopine dissolved in 10 ml of 0.01 M hydrochloric acid. For the air-dried sample the equivalent weight 297 was determined. The remaining part of the curve was obtained by titration with 1 M sodium hydroxide of 7.584 mg of anhydrous saccharopine suspended in 250 µl of water (M 276). The titration values were converted into equivalents of sodium hydroxide per mole of saccharopine.

Acetone titration of saccharopine. Because the amino acid is slightly soluble in acetone, the original method [6] could not be used but was substituted by a modification of the procedure described by Zamecnik *et al.*[7]

From an approximately 0.01 M aqueous solution of saccharopine 0.40 ml was transferred to a test tube (3 1/8″ × 1/2″) designed for the "EEL" colorimeter. To this amount were added 6.3 µl of 0.1 % naphthyl red in 96 % ethanol and a known volume of 0.1 M HCl in 96 % ethanol, followed by 3.60 ml of acetone. The optical density (D) of the solution was immediately read in an "EEL"-colorimeter provided with filter (OGRI). It is important that the addition of acetone and the reading of D are done as rapidly as possible, since the acetone solution becomes turbid after a few minutes.

A blank value was determined as follows: a mixture of 0.40 ml of water, 3.60 ml of acetone and 6.3 µl of naphthyl red was titrated, and a curve constructed of the colorimetric reading as a function of the amounts of added HCl (in µl). This curve was used to estimate the blank acid consumption equivalent to the determined D-value. Determination on three 0.40 ml samples of a saccharopine-solution containing 0.27 N/ml gave titration values of 85 µl of 0.1 M HCl, corresponding to a content of 1.08 equivalent per equivalent of nitrogen.

Paper electrophoresis. Mixtures of saccharopine and its transformation product were studied on Whatman No. 1 paper in 0.1 N citrate buffers and 0.1 and 1 N acetic acid at 3 V per cm. The apparatus used was constructed as described by Kunkel and Tiselius [8].

Isolation of the transformation product. An amount of 100 mg of saccharopine was treated with 6 N HCl at 125° for 96 h. Saccharopine and its conversion product were separated from HCl by absorption on a Zeo-Karb 225 (H^+) column and eluted with 1 M NH_3. The eluate was evaporated to dryness *in vacuo*, and the residue dissolved in 25 ml of water. The mixture was then separated by continuous electrophoresis in an "Elphor V" apparatus at pH 3.8, utilizing an acetate buffer (ionic strength 0.05) at 170 V and 14 mA. Schleicher and Schüll paper No. 2040b was employed. The solution was applied to the paper at a rate of 0.36 ml/h. The fractions were collected in 22 tubes (No. 1 at the anode). The solution was applied above tube No. 21. The fractionation was controlled by paper chromatography in butanol:acetic acid:water. Saccharopine was found to be present in the tubes 17, 18 and 19, and its conversion product in 14 and 15. The latter fractions were pooled (26 ml) and applied to a Zeo-Karb 225 (H^+) column. The transformation product was eluted with ammonia solution and evaporated to dryness, and the residue was dissolved in 0.1 M HCl and precipitated with 10 volumes of acetone. Analyses of the contaminating ammonium chloride were performed by determination of ammonia by the Conway-method and chloride as described by Brun [9]. A content of 6.8 % of NH_4Cl was found.

Micro-Kjeldahl analyses were performed with selenium as a catalyst.

Baker's yeast was generously supplied by *A/S Dansk Gæringsindustri* and brewer's yeast by the *Research Laboratory of the Carlsberg Breweries.* Microanalyses for C, H and N (Dumas) were performed by Mr. P. Hansen, The Chemical Laboratory of the University of Copenhagen. The authors wish to thank Prof. A. Kjær for help during the preparation of the manuscript and Dr. P. J. Christensen for the determinations of the ninhydrin colour yields. The assistance of Dr. A. Stenderup in the microbiological studies is gratefully acknowledged.

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