Monatshefte für Chemie 115, 1255—1267 (1984)

Monatshefte für Chemie
Chemical Monthly

Characterisation of Naringinase from *Aspergillus niger*

Michaela Roitner, Thomas Schalkhammer, and **Fritz Pittner***

Institut für Allgemeine Biochemie, Universität Wien, und
Ludwig-Boltzmann-Forschungsstelle für Biochemie, A-1090 Wien, Austria

(Received 24 May 1984. Accepted 9 July 1984)

Naringinase from *Aspergillus niger* shows α-*L*-rhamnosidase and β-*D*-glucosidase activity. The ratio of these enzymatic activities varies, depending on the protein concentration as well as the *pH*. The rhamnosidase activity is nearly independent of the *pH* in the range from 3 to 7, whereas glucosidase shows a distinct optimum which varies between *pH* 4 and *pH* 6 depending on its pretreatment. By gel filtration the enzyme complex can be separated into various oligomers, which are multiples of the smallest active subunit with a molecular weight of 95 000. The oligomers show either both enzymatic activities or mere rhamnosidase action. Protein fractions with glucosidase activity only could not be isolated. However in fractions with rhamnosidase activity only, the glucosidase activity could be restored by immobilisation. Glucosidase activity is related to the concentration of protein in solution, disappearing in very diluted solutions, where rhamnosidase is still active.

(Keywords: Enzyme characterisation; Naringinase)

Charakterisierung von Naringinase aus Aspergillus niger

Naringinase aus *Aspergillus niger* zeigt α-*L*-Rhamnosidase- und β-*D*-Glucosidaseaktivität. Das Verhältnis von Rhamnosidase- und Glucosidaseaktivität im Enzymkomplex kann sich verändern und hängt von der Proteinkonzentration und dem *pH* ab. Die Rhamnosidaseaktivität des Enzymkomplexes zwischen *pH* 3 und *pH* 7 ist nahezu konstant, während die Glucosidase ein deutliches Optimum besitzt, das allerdings je nach Vorbehandlung zwischen *pH* 4 und *pH* 6 schwanken kann. Durch Gelfiltration kann der Enzymkomplex in verschiedene Oligomere aufgetrennt werden. Alle Fraktionen sind Vielfache der kleinsten aktiven Einheit ($M = 95\,000$) und besitzen entweder beide Enzymaktivitäten oder nur Rhamnosidaseaktivität. Aktive Glucosidase allein konnte nicht isoliert werden. Durch Immobilisierung kann jedoch in Proteinfraktionen mit reiner Rhamnosidaseaktivität wieder totale Naringinaseaktivität induziert werden. Die Glukosidaseaktivität ist abhängig von der Proteinkonzentration und verschwindet in sehr verdünnten Lösungen, während die Rhamnosidase unter diesen Bedingungen aktiv bleibt.

* Dedicated to Professor Dr. *Karl Schlögl* at the occasion of his 60th anniversary.

Introduction

Naringinase from *Aspergillus niger* is an enzyme complex consisting of an α-*L*-rhamnosidase (EC 3.2.1.40) and a β-*D*-glucosidase (EC 3.2.1.21) capable of hydrolyzing various glycosides like naringin, poncirin and neohesperidin to their aglycons[1]. Only few data characterizing the enzyme complex are available: The glucosidase can be inactivated by treatment at 60 °C and *pH* 6.4 to 6.8 for 30 min[1]. The overall naringinase activity can be partly inhibited by fructose, glucose and sucrose, whereas rhamnose partly inhibits the rhamnosidase and glucose, galactose, maltose and mannose the glucosidase activity. Other monosaccharides showed no inhibitory effects[2,3]. *Dunlap* et al.[4] were able to separate a fungal naringinase preparation into individual glucosidase and rhamnosidase fractions by paper electrophoresis.

Naringinase was so far mainly used in the industry for debittering of citrus juices. Besides this technical application it can be used to remove rhamnose and glucose from various glycosides to obtain the aglycons. Therefore it seemed of interest to us to get more information on the molecular properties of the enzyme complex.

Experimental

Materials

The enzymes naringinase from *Aspergillus niger**, alcohol dehydrogenase, ovalbumin and serumalbumin were from Sigma, St. Louis, cytochrome c, lysozyme, malate dehydrogenase, lactate dehydrogenase, catalase, fructose-bisphosphate aldolase (muscle), pyruvate kinase and phosphorylase a from Boehringer, Mannheim.

2-Aminodiphenyl, acrylamide, tetramethyl ethylene diamine (*TEMED*), Dowex 1 × 4 were from Serva, Heidelberg; naringin, naringenin, Coomassie Brilliant Blue, Dextrane Blue, Dowex 50 × 1–100, controlled pore glass (100–200 mesh, mean pore diameter 253 A) from Sigma, bis-acrylamide from EGA, Steinheim-Albuch, Sephadex G 200 from Pharmacia, Uppsala, ammonium peroxodisulfate, sodium dodecylsulfate, silver nitrate, *Schiff*'s reagent, aniline phthalate, TLC plates silica gel 60F254 with concentrating zone, TLC cellulose plates, organic solvents and all buffer substances from Merck, Darmstadt.

Employed buffer solutions: *pH* 3–4: 0.2 *M* acetate buffer; *pH* 5–7: 0.15 *M* phosphate buffer.

Methods

1. Purification of naringinase preparations: Commercially available naringinase lyophylisate (100 mg) was suspended in 30 ml water, kept under gentle stirring at 4° C and the undissolved components removed by centrifugation. The soluble fraction was lyophilized again and stored for further use.

---

* The enzyme was kept in stock until 1983 and replaced since 1984 by naringinase from *penicillium* species.

The soluble protein of that lyophilisate turned out to be electrophoretically almost pure enzyme (at least 98% pure), since only traces of other protein could be found by silver staining. The concentration of the impurities was below the sensitivity of our quantitative protein test (Fig. 1)[5].

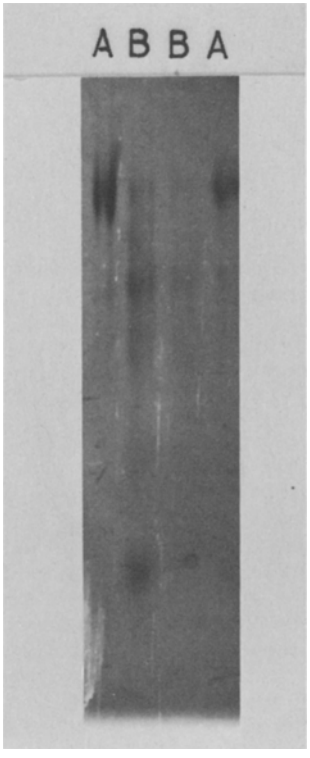


Fig. 1. Polyacrylamide gel electrophoresis of naringinase: *A* purified enzyme, *B* commercially available enzyme

2. Assay of naringinase as well as of its components rhamnosidase and glucosidase for activity was carried out following a method of *Habelt* and *Pittner*[6].

Batches of 2 mg naringinase in 6 ml buffer solution *pH* 3, 4, 5, 6, and 7 containing 3.4 μ-moles naringin were incubated at 37 °C for 30 min. Now aliquots of the incubation mixture were subjected to the spectrophotometric test. This assay allows to determine the amount of naringin, prunin and naringenin present in the incubation mixture by combining two spectrophotometric procedures: (a) Treatment with strong alkali to determine the amount of naringenin (at 310 nm) as well as the sum of naringin and prunin (at 375 nm). (b) Assay of the liberated aldohexoses with *o*-amino diphenyl. From the data thus obtained the amount of the remaining substrate as well as of the product at any given time and thus the enzymatic activities of the rhamnosidase and the glucosidase can be calculated.

3. Chromatographic detection of naringin, prunin and naringenin was carried out in order to have a qualitative control for the reaction, to check the purity of the compounds as well as to isolate prunin from incubation mixtures.

Aliquots (3 ml) of the incubation mixtures were extracted 3 times with 4 ml ethylacetate. The combined organic fractions containing the flavanone compounds were evaporated to dryness, redissolved in 100 µl ethylacetate and used for chromatographic separation on thinlayer KGF254 plates (Merck). The plates were developed in acetone—chloroform—water (80 : 20 : 4.8). The detection of the spots was carried out either under UV at 254 nm or by spraying with vanilline—H_2SO_4 according to *Le Rosen* et al.[7] and *Tyihak* et al.[8], or by insertion into a I_2 chamber. *Rf* (naringin) = 0.17; *Rf* (prunin) = 0.33; *Rf* (naringenin) = 0.64.

The separated compounds were eluted and assayed spectrophotometrically after treatment with 3 ml aqueous 4 *M* NaOH for 20 min. The absorbance was measured at 375 nm in the case of naringin or prunin fractions and at 310 nm with naringin fractions eluted from the thin layer plates.

4. Thin layer chromatography of the liberated sugars: The remaining aqueous solutions of procedure 2 were deionized with Dowex 1 × 4 and Dowex 50 × 1–100 ion exchange resins. The deionized solutions containing the liberated sugars were evaporated to dryness and redissolved in 100 µl water. Aliquots (10–30 µl) of the various batches were developed by thin layer chromatography on cellulose plates (Merck) with acetone—water (85 : 15). The chromatograms were treated with anilinephthalate[9].

Rf (rhamnose) = 0.7, *Rf* (glucose) = 0.26.

5. Immobilisation of naringinase on silane coated glass beads: Controlled pore glass beads (1 g) were coated with 3-aminopropyl triethoxy silane according to *Weetall*[10] and activated with an excess of 10% aqueous glutaraldehyde solution for 4 h at room temperature following a procedure described in [11]. For immobilisation of the enzyme protein the carefully washed glass beads were incubated on a shaker over night at room temperature with the respective amount of protein provided for immobilisation, dissolved in the various coupling buffers. The buffers as well as the ratio of the amount of glass beads and enzyme varied with the working conditions employed. Therefore further details are given in the chapter results. All immobilized enzyme preparations could be stored for months at 0 °C or room temperature without changing their properties.

6. Determination of protein content: The quantitative determination of immobilized protein was carried out on an amino acid analyzer or according to *Jacobs*[5] after acid hydrolysis with 6 *M* HCl in a sealed tube for 22 h at 110 °C. In the same manner the protein content of native protein solutions was measured. For qualitative tests after elution from Sephadex columns spectrophotometric measurement at 280 nm was employed.

7. Estimation of molecular weights was done by gel chromatography on Sephadex G 200[12], using cytochrome c, lysozyme, malate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase, ovalbumin, catalase and Dextran Blue as markers.

8. Polyacrylamide gel electrophoresis was carried out on gel plates containing 7.5% acrylamide prepared by a slightly modified procedure according to *Maurer*[13]. The dissociation of the naringinase proteins was achieved by treatment with sodium dodecyl sulfate[14,15].

The molecular weights of the enzyme subunits were determined with the help of the following standard marker proteins: cytochrome c, lysozyme, lactate dehydrogenase, fructose bisphosphate aldolase (muscle), ovalbumin, pyruvate kinase, catalase, serum albumin and phosphorylase a. For *staining of the bands* Coomassie Brilliant Blue R and silver staining according to *Wray* et al.[16] was used.

9. Estimation of enzyme activity on polyacrylamide gels: The gel was cut into cross sections of 1 cm width. The various cross sections were ground into small particles and suspended in 4 ml 1 *M* acetate buffer *pH* 4. The incubation was started by addition of 1 ml aqueous naringin solution (0.5 mg naringin/ml) and incubated over night at 37 °C on a shaker. The products formed were assayed as described above[6]. Because of the long incubation time we took the results thus obtained to be only semiquantitative.

Results

I. Mechanism of the Naringinase Action

The reports in the literature on the total action of the enzyme complex from various sources differ considerably concerning the sequence of the reaction steps for the cleavage of the sugar part[17–20]. Besides the steps shown in Scheme 1, a degradation of naringin to naringenin and the disaccharide rutinose followed by the cleavage of the sugar to rhamnose and glucose might be possible. To prove this with our type of naringinase chromatographic determinations of the reaction products and intermediates—including the sugars—were carried out as described in the methods section.

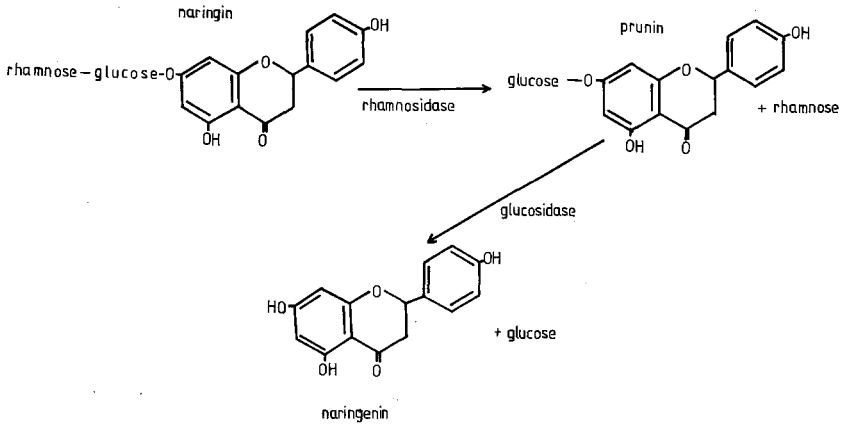
Only rhamnose and glucose could be found between *pH* 3 and 6, at *pH* 7 only rhamnose was detected, but rutinose never appeared. These results of the sugar chromatography are in accordance with the quantitative data we obtained from flavanone chromatograms and the spectrophotometric assay[6], which only gives exact results, when no rutinose is formed (Table 1).

Table 1. *Ratio of naringin, prunin and naringenin formed by the action of naringinase depending on pH estimated by (I): Spectrophotometric assay according to* [9]*; (II): Chromatography followed by spectrophotometric measurement*

pH	Mole fractions of					
	naringin		prunin		naringenin	
	I	II	I	II	I	II
3	0.25	0.27	0.30	0.34	0.40	0.39
4	0	0	0.36	0.34	0.64	0.68
5	0	0	0	0	1.00	1.00
6	0.04	0.05	0.17	0.17	0.79	0.78
7	0	0	1.00	1.00	0	0

Under our working conditions the action of naringinase follows Scheme 1 and is in accordance with the findings of *Shigetaka Okada* et al.[17] but does not support the data of other authors[18]. We will come back to this problem in more detail in the discussion.

Scheme 1. Degradation of naringin to naringenin by the action of naringinase



II. Determination of the pH Optimum

The naringinase action was tested at various *pH* following the spectrophotometric assay described above[6]. The results are given in Fig. 2.

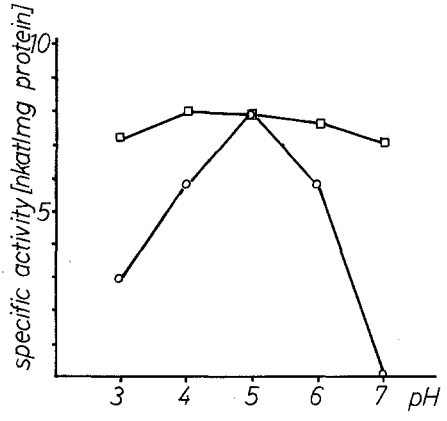


Fig. 2. Average specific activities of rhamnosidase and glucosidase obtained from the data in Table 1. □—□ : rhamnosidase; ○—○ : glucosidase

The data show that in the active holoenzyme complex the rhamnosidase activity is rather independent on the *pH* within the range of 3 to 7. The glucosidase activity has a distinct maximum at *pH* 5. The *pH* dependence of glucosidase was the same when prunin was used as a substrate instead of naringin.

III. Behaviour of Naringinase Dependent on the Protein Concentration in the Incubation Mixture

a. Native Enzyme: Varying amounts of native naringinase (0.01–0.1 mg enzyme) dissolved in 3 ml acetate buffer *pH* 5 or phosphate buffer *pH* 7, respectively, were incubated with 1.7 μ-moles naringin for 30 min at 37 °C. The reaction mixture was assayed for naringin, prunin and naringenin content (Fig. 3). For comparison the specific activities are

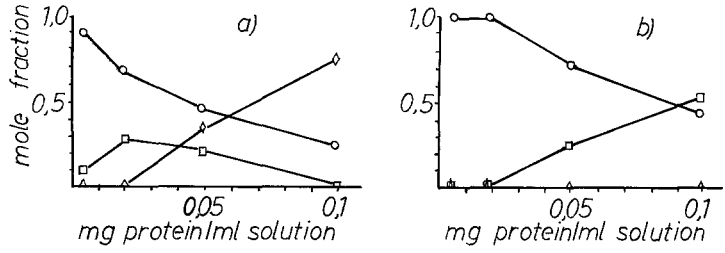


Fig. 3. Amounts of naringin, prunin and naringenin present in the assay mixture after an incubation time of 30 min depending on the protein concentration. *a* incubation at *pH* 5; *b* incubation at *pH* 7. □—□ : naringin; ○—○ : prunin; ◇—◇ : naringenin

given in Fig. 4 (*a, b*). In the range of the *pH* optimum (*pH* 5) the specific activity of the total naringinase activity decreases with decreasing protein concentration. The most diluted fraction showed only rhamnosidase activity. With increasing protein concentration more naringenin was formed and less intermediate prunin could be found, indicating that the glucosidase activity increases, whereas the rhamnosidase activity gradually declines.

b. Naringinase Immobilized on Glutaraldehyde Activated Glass Beads: Various concentrations of naringinase (0.06, 0.12, 0.25, and 0.5 mg/20 ml phosphate buffer *pH* 7) were immobilized on glutaraldehyde activated amino glass beads (300 mg) as described in the methods section.

These small amounts of protein were chosen to study the enzyme complex under very diluted conditions, where steric hindrances of the immobilisate may be excluded[11]. Aliquots of the immobilisates thus obtained (20 mg) were assayed for activity with naringin. The content of immobilized protein was determined according to *Jacobs*[5]. The results are given in Table 2 and Fig. 4 *c*.

Only the immobilisate with the highest amount of protein shows total naringinase activity, in all other preparations only rhamnosidase activity could be found.

Table 2. *Action of immobilizes naringinase dependent on the amount of protein bound*

Amount of protein bound		Mole fractions of [a]		
used for coupling [mg]	[mg/g wet beads]	naringin	prunin	naringenin
0.5	1.11	0.41	0.33	0.26
0.25	0.51	0.53	0.47	0
0.12	0.31	0.74	0.26	0
0.06	0.16	0.77	0.23	0

[a] After 30 min of incubation time.

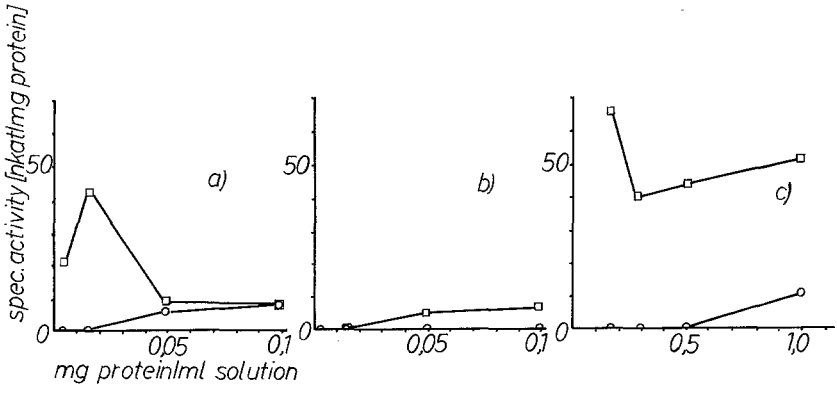


Fig. 4. Changes in specific activity of rhamnosidase and glucosidase dependent on the protein concentration. *a* native enzyme incubated at *pH* 5; *b* native enzyme incubated at *pH* 7; *c* immobilized enzyme assayed at *pH* 5. □—□ : rhamnosidase; ○—○ : glucosidase

IV. Gel Chromatography on Sephadex and Gel Electrophoresis under Various Conditions

1. Gel Chromatography on Sephadex Columns

a. Estimation of Molecular Weights: To estimate the molecular weight of the naringinase complex or fractions of it, gel filtration on Sephadex G 200 (column size 2 × 80 cm) was carried out at *pH* 5, 7, and 9 according to the procedure described in the methods section. Fractions of 3 ml were collected and aliquots assayed for protein content and enzymatic activities. The protein fractions eluted from the columns were tested for activity as usual with naringin, but prunin was also used as a substrate to detect fractions with glucosidase activity only. Only fractions with total naringinase activity or with rhamnosidase activity only could be found. No isolated glucosidase activity was detected under our test conditions.

The smallest enzyme protein obtained by these experiments which showed total naringinase activity had a molecular weight of 95 000. All other active enzyme fractions obtained were multiples of this molecular weight: 190 000 (2 ×), 285 000 (3 ×), and 380 000 (4 ×), all showing total naringinase activity.

It should be mentioned that glycoproteins containing more than 5% of carbohydrate might behave as larger proteins than their equal molecular weights would indicate. But the deviations seem to be generally within a definite range, so that even without a precise correction factor the method can still be usefully applied[21–23].

b. Induction of Total Naringase Activity with the Help of Immobilisation: All protein fractions obtained from the gel chromatography on Sephadex columns with rhamnosidase activity only were separatedly immobilized to controlled pore glass as described in the methods section. To obtain measurable results the various fractions of immobilisate were combined, resulting in 200 mg wet immobilisate containing 0.15 mg bound protein. Because of the low protein concentration the assay for activity was only possible qualitatively with the help of thinlayer chromatography on TLC plates Silicagel 60F254 with concentrating zone. Not only prunin, but also naringenin could be detected in the incubation mixture, showing that the total naringinase activity could be reactivated by the immobilisation step.

c. Sodiumdodecylsulfate Gel Electrophoresis: The naringinase fractions with molecular weights of 95 000, 190 000, 285 000, and 380 000 were each subjected to SDS gel electrophoresis[19]. All naringinase fractions tested showed only one protein band with a molecular weight of 95 000 ± 8 000, which seems to be the smallest active subunit (Fig. 5).

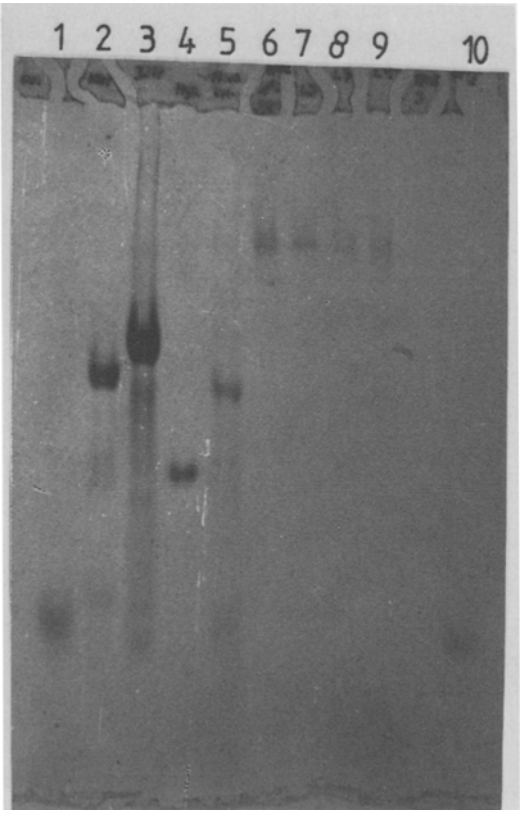


Fig. 5. SDS gel electrophoresis of naringinase fractions of different molecular weights. *1* cytochrome c; *2* catalase; *3* bovine serum albumin; *4* alcohol dehydrogenase; *5* α-amylase; *6* naringinase (fraction $M = 95\,000$); *7* naringinase (fraction $M = 190\,000$); *8* naringinase (fraction $M = 285\,000$); *9* naringinase (fraction $M = 380\,000$); *10* cytochrome c

2. Gel Electrophoresis on 7.5% Polyacrylamide Gel Plates

The naringinase fraction from Sephadex G-200 with a molecular weight of 380 000 was subjected to gel electrophoresis under varying *pH* conditions (*pH* 5, *pH* 8.3, and *pH* 10). In addition to that electrophoresis at *pH* 8.3 in presence of 2% glucose or 2% rhamnose respectively, was carried out. From each plate a small longitudinal section (1 cm width) was qualitatively assayed for protein bands with silver staining[16]. The remaining gel was assayed for enzymatic activity at the *pH* optimum with naringin and prunin as substrates as lined out in the methods section. The data are given in Table 3.

Table 3. *Gel electrophoresis of naringinase on 7.5% polyacrylamide gel plates*

Conditions during the separation	*Rf*[a]	Mole fractions[b] of		
		naringin	prunin	naringenin
pH 5	0.05	0	0	1.00
	0.12	0.80	0.20	0
pH 8.3	0.04	0	0	1.00
	0.08	0	0.20	0.80
	0.12	0.20	0.40	0.40
pH 8.3	0.16	0	0.20	0.80
2% glucose	0.30	0	0	1.00
	0.45	0.70	0.30	0
	0.53	0.80	0.20	0
	0.72	0.90	0.10	0
pH 8.3	0.10	0.30	0.23	0.47
2% rhamnose	0.16	0	0	1.00
	0.28	0.53	0.26	0.21
	0.33	0.92	0.08	0
pH 10	0.07	0	0.70	0.30
	0.14	0.60	0.40	0
	0.20	0.80	0.20	0

[a] Migration length of protein/migration length of frontmarker.
[b] After 30 min of incubation time.

Dependent on the *pH* of the separation buffer 2 to 3 protein bands were found. All of them showed either total naringinase or only rhamnosidase activity. The presence of glucose or rhamnose caused in increase in the number of protein bands up to 5. The protein bands which migrated only a small distance during the electrophoresis showed the highest activity with respect to the formation of the end product naringenin. Faster migrating protein bands were less active or showed only rhamnosidase action with decreasing activity.

Discussion

Different opinions exist about the action of the enzyme complex on its substrate naringin which is a 4',5,7-trihydroxyflavanone 7-rhamnoglucoside: *Kiyoshi Kishi*[18] reports that naringenin and the disaccharide rutinose are formed first by the glucosidase action, followed by the decomposition of the rutinose to glucose and rhamnose with the rhamnosidase. On the other hand *Smythe* and *Thomas*[20] reports that prunin (naringenin 7-glucoside) is formed between *pH* 2.5 and 4.5 by the

action of naringinase. *Shigetaka Okada* et al.[17] found that naringin is decomposed first to prunin and rhamnose followed by the degradation to naringenin and glucose.

The reinvestigation of this problem with the help of our spectrophotometric and chromatographic assay showed only formation of prunin, glucose and rhamnose as intermediates, but rutinose never could be found. This is in accordance with the findings of *Shigetaka Okada* et al.[17], but does not support those of *Kiyoshi Kishi*[18]. Of course we cannot exclude definitely that small amounts of rutinose are liberated by the glucosidase and are degraded immediately after formation. Maybe the mechanism of action of naringinase from other sources than *Aspergillus niger* is different. Studies to elucidate this problem are in progress.

The behaviour of naringinase dependent on the protein concentration in the incubation mixture shows that optimum concentrations of the enzyme are necessary for optimum overall naringinase activity at the *pH* optimum leading to an optimum ratio of rhamnosidase to glucosidase. It could be that the enzyme complex dissociates or associates depending on the protein concentration. Below a protein content of 0.05 mg/ml incubation mixture the enzyme was inactive, showing that a definite minimum enzyme concentration is necessary for optimum activity. Supported also by our findings described in chapter IV of the results section glucosidase seems to need a defined molecular dimension of the oligomer to show optimal activity.

These results support the assumption that the enzyme complex can exist in various active, aggregated forms which vary with respect to the ratio of rhamnosidase and glucosidase and behave also differently to changes in *pH* or addition of effectors like glucose and rhamnose.

The splitting of active protein bands with the help of gel electrophoresis shows again that rearrangements of active aggregates occur very easily depending on various parameters. Small amounts of glucose and rhamnose could be shown to promote further splitting into several active fractions. These 2 sugar components are constantly formed during the naringinase action and their increasing amount may constantly change the enzyme proteins present in the incubation mixture.

The capability of association or dissociation under various conditions is already well known from the literature, especially with glycoproteids and glycoenzymes (see for example [24,25]). Studies concerning the glycoprotein nature of naringinase are in progress and will be published soon.

All our results seem to indicate that naringinase from *Aspergillus niger* is one single enzyme protein with two active sites, one for *α-L*-rhamnosides and the other for *β-D*-glucosides. The enzyme forms oligomers and the ratio of the two activities is dependent on various physical influences.

Acknowledgements

The authors thank Prof. Dr. *O. Hoffmann-Ostenhof* and Prof. Dr. *H. Ruis* for critically reviewing the manuscript.

References

[1] *Shintaro Kamiya, Sachiko Esaki, Misao Hama,* Agr. Biol. Chem. (Tokyo) **31**, 142 (1967).
[2] *Danji Nomura, Keiji Akiyama,* Nippon Shokuhin Kogyo Gakkaishi **11**, 267 (1964).
[3] *Shigetaka Okada, Mayumi Yano, Junichiro Fukumoto,* Nippon Nogei Kagaku Kaishi **38**, 246 (1964).
[4] *Dunlap W. J., Hagen R. E., Wender S. H.,* J. Food Sci. **27**, 597 (1962).
[5] *Jacobs S.,* Nature **183**, 262 (1959).
[6] *Habelt K., Pittner F.,* Analyt. Biochem. **134**, 393 (1983).
[7] *Le Rosen A. L., Moravek R. T., Carlton J. K.,* Anal. Chem. **24**, 1335 (1952).
[8] *Tyihak E., Vaguifalvi D., Hagony P. L.,* J. Chromatogr. **11**, 45 (1963).
[9] Anfärbereagenzien für Dünnschicht- und Papierchromatographie. E. Merck. Darmstadt: 1970.
[10] *Weetall H.,* in: Methods Enzymol., Vol. 44 (*Mosbach K.,* ed.), p. 134. New York: Academic Press. 1976.
[11] *Pittner F., Miron T., Pittner G., Wilchek M.,* J. Solid Phase Biochem. **5**, 167 (1980).
[12] *Andrews P.,* Biochem. J. **96**, 595 (1965).
[13] *Maurer H. R.,* Diskelektrophorese. Berlin: W. de Gruyter. 1968.
[14] *Laemmli U. K.,* Nature **227**, 680 (1970).
[15] *Weber K., Pringle J. R., Osborn M.,* in: Methods Enzymol. Vol. 26 c (*Hirs C. H. W., Timasheff S. N.,* eds.), p. 3. New York: Academic Press. 1973.
[16] *Wray W., Boulikas T., Wray V. P., Hancock R.,* Analyt. Biochem. **118**, 197 (1981).
[17] *Shigetaka Okada, Mayumi Yano, Junichiro Fukumoto,* Hakko Kyokaishi **22**, 371 (1964).
[18] *Kiyoshi Kishi,* Kagaku to Kogyo **33**, 185 (1959).
[19] Polyacrylamide Gel Electrophoresis Laboratory Techniques (revised ed.), p. 54. Upsala: Pharmacia Fine Chemicals.
[20] *Smythe C. V., Thomas D. W.,* U.S. 2.950.974 (1960).
[21] *Andrews P.,* Biochem. J. **96**, 595 (1965).
[22] *Andrews P.,* in: Protides of the Biological Fluids, Vol. 14 (*Peters H.,* ed.), p. 573. Amsterdam: Elsevier. 1967.
[23] *Andrews P.,* in: Methods of Biochemical Analysis, Vol. 18 (*Glick D.,* ed.), p. 33. New York: Interscience, J. Wiley & Sons. 1970.
[24] *Pazur J. H., Knull H. R., Simpson D. L.,* Biochem. Biophys. Res. Comm. **40**, 110 (1970).
[25] *Bettelheim F. A.,* Biochim. Biophys. Acta **236**, 702 (1971).

Verleger: Springer-Verlag KG, Mölkerbastei 5, A-1010 Wien. — Herausgeber: Österreichische Akademie der Wissenschaften, Dr.-Ignaz-Seipel-Platz 2, A-1010 Wien, und Gesellschaft Österreichischer Chemiker, Eschenbachgasse 9, A-1010 Wien. — Redaktion: Währinger Straße 38, A-1090 Wien. — Hersteller: Adolf Holzhausens Nachfolger, Kandlgasse 19-21, A-1070 Wien. — Verlagsort: Wien. — Herstellungsort: Wien. — Printed in Austria.