INVESTIGATION ON THE β-GALACTOSIDASE OF ALFALFA SEED EMULSIN

by

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In a very interesting paper HILL[1] has described the isolation and the investigation of alfalfa seed emulsin, showing that this enzyme catalyses the hydrolysis of β-galactosides but not of β-glucosides. From this statement HELFERICH[2] draws the conclusion that two different β-galactosidases must exist, one, identical with β-glucosidase, is found e.g., in almond emulsin, and another, different from β-glucosidase, is found in alfalfa seed emulsin and in coffee emulsin (HELFERICH AND VORSATZ[3]).

HELFERICH and his school do not, however, investigate the glycosidases very thoroughly. No determination e.g., of the affinity constants, K_{m_x}, i.e., the dissociation constants of the compounds formed by the glycosidase and its substrate, or the products of hydrolysis of the substrate, has been carried out. That is why we have undertaken a closer study of the β-galactosidase of alfalfa seed emulsin.

In a previous paper (VEIBEL AND LILLELUND[4]) one of us has reported on a thorough investigation of the ß-glucosidase of almond emulsin with determination of K_m, K_{m1} and K_{m2}-values for o-cresol-β-d-glucoside, glucose and o-cresol. Later (VEIBEL, WANGEL AND ØSTRUP[5]) a quite analogous examination of the β-galactosidase of almond emulsin has been made. These investigations have shown that even if the two enzymes in many regards behave as if they were identical, still so many and such significant differences are found that it becomes a matter of definition whether the two glycosidases are regarded as identical or if two different glycosidases, one a β-glucosidase, the other a β-galactosidase, are to be presumed. This latter assumption seems, all facts taken in account, the most probable.

VEIBEL, MØLLER AND WANGEL[6] investigated the β-glycosidase content in milk-sugar yeast emulsin and were able to show the presence of a β-glucosidase whereas no β-galactosidase could be found, a rather surprising result since HOFMANN[7] AND NEUBERG AND HOFMANN[8] have shown previously that milk-sugar yeast emulsin is able to catalyse the hydrolysis of the β-galactoside milk-sugar. If the result of VEIBEL, MØLLER AND WANGEL can be reproduced it means either that β-glucosidase and β-galactosidase are two different enzymes, or that not only two different β-galactosidases exist, one identical with, the other different from β-glucosidase, but also two different β-glucosidases, the one identical with, the other different from β-galactosidase.

In order to examine more closely the assumption of two different β-galactosidases formulated by HELFERICH we have now investigated alfalfa seed emulsin in a manner similar to that described for the β-galactosidase of almond emulsin (VEIBEL, WANGEL, AND ØSTRUP[5]). The result of this investigation is that rather large differences

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between alfalfa seed emulsin β-galactosidase and almond emulsin β-galactosidase seem to exist. For example the K_m is for almond emulsin β-galactosidase 0.054 (at p_H 3.6-5.6), for alfalfa seed emulsin β-galactosidase immeasurably small (at p_H 2.2-4.2). In both cases the hydrolysis of o-cresol-β-d-galactoside in phosphate-citrate buffer was followed.

K_{m1} (galactose) for almond emulsin β-galactosidase 3-8 times K_m (at p_H 3.6-5.6), for alfalfa seed emulsin β-galactosidase only 0.5-0.7 times K_m (at p_H 2.2-4.2).

K_{m1} (glucose) for almond emulsin β-galactosidase 0.41, not very different from the value found for almond emulsin β-glucosidase. For alfalfa seed emulsin β-galactosidase, on the contrary, the value is oo as no inhibition at all is caused by addition of glucose.

K_{m2} (o-cresol) for almond emulsin β-galactosidase is 0.5-1 time K_m (at p_H 3.6-5.6), for alfalfa seed emulsin β-galactosidase 4.6-6.7 times K_m (at p_H 2.2-4.2).

These figures mean that while the affinity between almond emulsin and o-cresol-β-d-galactoside is slighter than between almond emulsin and o-cresol-β-d-glucoside, the affinity between alfalfa seed emulsin and o-cresol-β-d-galactoside is so great that no reliable determination of the dissociation constant is possible, at least with the technique used here.

The affinity between almond emulsin and galactose is considerably slighter than between almond emulsin and o-cresol-β-d-galactoside. Galactose, consequently, causes only a slight inhibition of the hydrolysis. For alfalfa seed emulsin, on the contrary, the affinity to galactose is still greater than to o-cresol-β-d-galactoside and galactose is, therefore, a powerful (competitive) inhibitor of the hydrolysis.

The affinity between almond emulsin and o-cresol is considerably greater than between almond emulsin and o-cresol-β-d-galactoside. o-Cresol, therefore, shows a very considerable inhibition of the hydrolysis, an inhibition which at small p_H-values is certainly not exclusively competitive. The affinity between alfalfa seed emulsin and o-cresol, on the contrary, is so slight that an addition of o-cresol, even in such a quantity that the molar concentration of o-cresol and o-cresol-β-d-galactoside is the same, does not cause any measurable inhibition of the hydrolysis.

These differences may possibly be explained by differences in the colloidal carriers of the two enzymes, the prostetic groups of which are thought to be identical. This explanation also allows for the differences found in the p_H-optimum, which is at p_H 4.8 for almond emulsin β-galactosidase, at p_H 3.4 for alfalfa seed emulsin β-galactosidase (phosphate-citrate buffer).

The calculation of k_3 in the usual way (VEIBEL AND LILLELUND [4, 9]) gives tolerably constant values except at p_H 2.6 where the addition of galactose or o-cresol in concentrations larger than the galactoside-concentration causes abnormally small k_3-values.

The values given in Table VII are not the real k_3-values as the „salicin factor" of the enzyme preparation cannot be determined in the usual manner with salicin as substrate, but k_3 (sal. f.). If, however, the β-galactosidase of almond emulsin is regarded as a standard for the β-galactosidatic action, then a standardization of other β-galactosidase preparations in relation to almond emulsin β-galactosidase may be made, and if the enzymic force of this latter enzyme arbitrarily is fixed to the same value as that of its β-glucosidase, values for all β-galactosidase preparations may be calculated. This equals the calculation of the „salicin value" from measurements of the hydrolysis-velocity of o-cresol-β-d-glucoside as proposed by VEIBEL AND LILLELUND [10]. VEIBEL, WANGEL

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AND ØSTRUP [5] have determined the k_3-values at different p_H for the hydrolysis of o-cresol-β-d-galactoside with almond emulsin. By comparing the values of k_3 (sal. f.) found for alfalfa seed emulsin as indicated above with the corresponding k_3-values for almond emulsin of sal. f. 0.078 the sal. f.-value of the alfalfa seed emulsin is calculated to 0.012 if the k_3-values at p_H 3.4 are compared, or 0.007 if alfalfa seed emulsin at its p_H optimum, 3.4, is compared with almond emulsin at its p_H-optimum, 4.8. The enzymic force of the alfalfa seed emulsin is consequently 1/6-1/11 of the enzymic force of the almond emulsin used.

If the compound protein-prostetic group is able to dissociate (experiments of HELFERICH and co-workers [11] do not indicate this, whereas MALAGUZZI-VALERI [12] is of the opinion that his experiments do indicate such a dissociation) it might be possible that a solution which at the same time contains almond emulsin and alfalfa seed emulsin would show a catalytic effect on the hydrolysis of glycosides which was not merely the sum of the action of the two enzymes.

We have examined, therefore, the hydrolysis of o-cresol-β-d-glucoside and o-cresol-β-d-galactoside in presence of known mixtures of the two enzyme preparations, both at p_H 3.4 and at p_H 4,8, and have compared the velocity constants found with those calculated from the known k/e-values and e-values. The result was that for o-cresol-β-d-galactoside the constants found were slightly inferior to those calculated. For o-cresol-β-d-glucoside at the p_H-optimum for almond emulsin the calculated and the found velocity constants were identical, whereas the constants found at the p_H-optimum for alfalfa seed emulsin were clearly inferior to the constants calculated, i.e., the alfalfa seed emulsin has acted as an inhibiting impurity.

From these experiments no conclusion with regard to the dissociability of the enzyme-system can be drawn.

EXPERIMENTAL PART

Substrates. o-Cresol-β-d-galactoside was prepared as indicated by HELFERICH AND SCHMITZ-HILLEBRECHT [13] and showed the constants indicated by VEIBEL, WANGEL AND ØSTRUP [5], M. P. 193—195°, $[\alpha]_D^{20^\circ} = -40.5^\circ$. It contained 1 mol crystal-water.

o-Cresol-β-d-glucoside was prepared by current methods and showed the constants indicated in the literature.

Enzyme. The alfalfa seed emulsin was prepared from 1 kg seeds of Italian alfalfa, most kindly placed at our disposal by Director BAGGE ANDERSEN, Faellesforeningen for Danmarks Brugsforeninger. Also here we wish to thank Mr BAGGE ANDERSEN for the valuable assistance he has given us.

From the seeds the enzyme preparation was prepared as indicated by HILL [1]. The preparation „1 time purified" was used.

Buffer-solutions. In most of the experiments buffers prepared by mixing 0.2 m solution of disodium phosphate with a 0.1 m solution of citric acid (MC. ILVAINE [14]) were used. Only the determination of the p_H-optimum is made both in phosphate-citrate-buffered solutions and in solutions buffered with sodium citrate-hydrochloric acid-solutions as indicated by SØRENSEN [15].

Method. As usual, samples to 5 ml were taken, the enzymic action in the samples was suppressed by mixing with 1 ml 20% K_2CO_3-solution and the rotation determined after 2-3 hours (Mutarotation).

The specific rotation of the galactoside and of galactose at p_H 10.5-10.6 (the p_H of the samples after mixing them with K_2CO_3-solution) were determined in separate experiments.

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1. *Comparison of the velocity of hydrolysis of o-cresol-β-d-glucoside and o-cresol-β-d-galactoside.*

TABLE I

GLYCOSIDE SOLUTIONS 0.0400 m. EMULSIN 0.0546 g IN 50 ml. p_H 4.0. 30°.

t min	o-Cresol-β-d-glucoside			o-Cresol-β-d-galactoside		
	α	c—x	k'.10⁴	α	c—x	k'.10⁴
0	—1.30	1.95	—	—0.73	1.72	—
60	—1.30	1.95	—	—0.57	1.56	7.1
240	—1.29	1.94	—	—0.15	1.14	7.4
480	—1.28	1.93	—	+0.20	0.79	7.0
1440	—1.30	1.95	—	+0.73	0.26	5.7
		average	0		average	7

It is seen from the table that whereas the galactoside is hydrolyzed with a measurable velocity, no hydrolysis whatever of the glucoside has taken place within the 24 hours of the experiment. The maximum value of the velocity constant is then about 1/100 of the velocity constant of the galactoside, k/e therefore $\leq 1.3.10^{-4}$. VEIBEL AND LILLELUND [4] have shown that k/e (sal. f.) for almond emulsin β-glucosidase at p_H 4.0 is 609.10^{-2} and consequently a maximum sal. f.-value for a β-glucosidase present in alfalfa seed emulsin is about 2.10^{-5}, i.e., the preparation is practically inactive as β-glucosidase.

2. *Determination of the p_H-optimum of the β-galactosidase.*

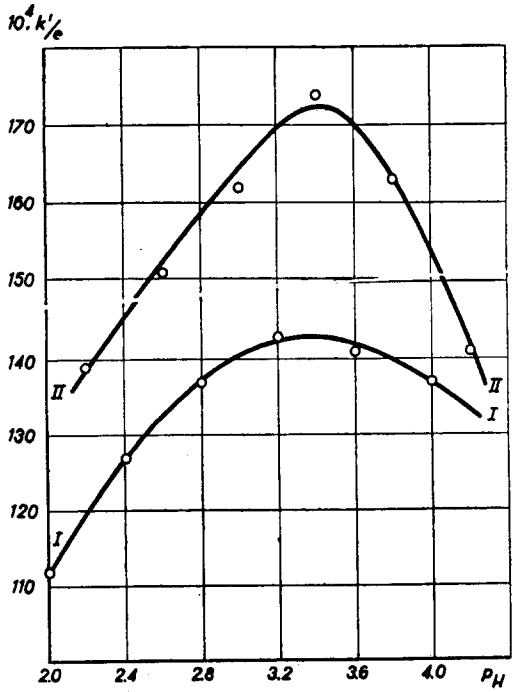

Fig. 1.
o-Cresol-β-d-galactoside. p_H-optimum I Sodium citrate-hydrochloric acid buffer II Phosphate-citrate buffer.

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TABLE II

a. In sodium citrate-hydrochloric acid buffer.

pH	2.0	2.4	2.8	3.2	3.6	4.0
$10^4 \cdot k'/e$	112	127	137	143	141	137
$10^4 \cdot k_3$ (sal. f.)	4.4	5.5	5.8	6.8	6.6	6.3

b. In phosphate-citrate buffer.

pH	2.2	2.6	3.0	3.4	3.8	4.2
$10^4 \cdot k'/e$	139	151	162	174	163	141
$10^4 \cdot k_3^2$ (sal. f.)	5.6	6.5	4.7	8.3	8.0	6.5

Table II and Fig. 1 show that the p_H-optimum in both buffer-systems is at about pH 3.4.

3. *Determination of* K_m.

The experimental results are recorded in Table III. The substrate concentration has been varied from 0.01-0.10 m. In all experiments phosphate-citrate buffers have been used.

The figures in the table show that the affinity between the enzyme and the substrate is so great that already at 0.01 m substrate the enzyme is completely bound to the substrate, as the initial velocity of hydrolysis, as measured by the alteration in rotation in 90 min, is independent of the substrate concentration, within the limits of error which must be put at $\pm$ 0.02° here. That means that a maximum value of K_m is 0.001 as this K_m-value allows the recognition of a difference in the initial velocity

TABLE III

DETERMINATION OF K_m

t min	c =	0.0100	0.0200	0.0400	0.0600	0.0800	0.1000 m
	a. pH 2.6						
90		0.14	0.14	0.11	0.12	0.14	0.14
180		0.22	0.29	0.30	0.29	0.29	0.29
270	x	0.29	0.35	0.40	0.38	0.41	0.41
360		0.31	0.43	0.48	0.47	0.51	0.53
∞		0.43	0.86	1.72	2.58	3.44	4.29
	b. pH 3.4						
90		0.16	0.19	0.17	0.15	0.13	0.15
180		0.26	0.32	0.35	0.33	0.29	0.34
270	x	0.32	0.44	0.48	0.48	0.47	0.50
360		0.34	0.50	0.59	0.61	0.61	0.61
∞		0.43	0.86	1.72	2.58	3.44	4.29
	c. pH 4.2						
90		0.16	0.13	0.12	0.10	0.12	0.12
180		0.23	0.26	0.26	0.28	0.23	0.25
270	x	0.29	0.38	0.40	0.41	0.38	0.41
360		0.32	0.45	0.51	0.52	0.54	0.54
∞		0.43	0.86	1.72	2.58	3.44	4.29

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of hydrolysis in 0.01 m galactoside solution (90% of the enzyme bound) and in 0.10 m galactoside solution (99% of the enzyme bound). K_m is, therefore, with the technique used here, immeasurably small.

4. *Determination of* K_{m1}.

Table IV gives the material. Substrate 0.0400 m o-cresol-β-d-galactoside. Phosphate-citrate buffer. 30°.

TABLE IV

DETERMINATION OF K_m/K_{m1}

$C_{galactose}$	pH 2.6 $10^4 k'_{obs}/e$	$\frac{k/k_h-1}{c_{Gal.}}$	$\frac{K_m}{K_{m1}}$	pH 3.4 $10^4 k'_{obs}/e$	$\frac{k/k_h-1}{c_{Gal.}}$	$\frac{K_m}{K_{m1}}$	pH 4.2 $10^4 k'_{obs}/e$	$\frac{k/k_h-1}{c_{Gal.}}$	$\frac{K_m}{K_{m1}}$
0.00	129	—		163	—		130	—	
0.01	92	39		112	46		96	35	
0.02	77	35		82	49		84	28	
0.04	43	50	2	64	42	2	61	29	1.5
0.08	31	38		29	58		31	40	
0.12	12	74		19	63		17	56	
	average	47		average	52		average	38	

As the K_m-value is immeasurably small, the usual expression for the calculation of $K_{m1} : K_{m1} = \frac{K_m \cdot c_{galactose}}{(K_m + c)\,(k/k_h - 1)}$, cannot be used. As, however, in the denominator K_m may be regarded as infinitesimal as compared with c the expression may be transcribed to $\frac{K_m}{K_{m1}} = \frac{c\,(k/k_h - 1)}{c_{galactose}}$, and consequently k_3 may be calculated. The same expression holds good for K_m/K_{m2}, $c_{o\text{-}cresol}$ being substituted for $c_{galactose}$.

5. *Determination of* K_{m2}.

Table V gives the material. Substrate 0.0400 m o-cresol-β-d-galactoside. Phosphate-citrate buffer. 30°.

TABLE V

DETERMINATION OF K_m/K_{m2}

C_{cresol}	pH 2.6 $10^4 k'_{obs}/e$	$\frac{k/k_h-1}{c_{Cr.}}$	$\frac{K_m}{K_{m2}}$	pH 3.4 $10^4 k'_{obs}/e$	$\frac{k/k_h-1}{c_{Cr.}}$	$\frac{K_m}{K_{m2}}$	pH 4.2 $10^4 k'_{obs}/e$	$\frac{k/k_h-1}{c_{Cr.}}$	$\frac{K_m}{K_{m2}}$
0.00	144	—		155	—		120	—	
0.01	138	3.7		148	5.0		108	10.2	
0.02	137	2.3		137	6.5		110	4.1	
0.04	131	2.5	0.15	140	2.8	0.17	107	3.0	0.22
0.08	98	5.9		119	3.8		89	4.3	
0.12	61	(11.3)		106	3.7		75	4.9	
	average	3.6		average	4.4		average	5.3	

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In Table VI and in Fig. 2 we have collected the results of these investigations and indicated the value of the correction-term in the expression for k_3:

$k_3 = k_{obs}\ (K_m + c + (K_m/K_{mh} \cdot c_h + K_m/K_{m1} + K_m/K_{m2} - 1)\ x)/e$ (sal. f.)

at different p_H-values. The boldfaced values are determined directly, the others are determined by graphical interpolation.

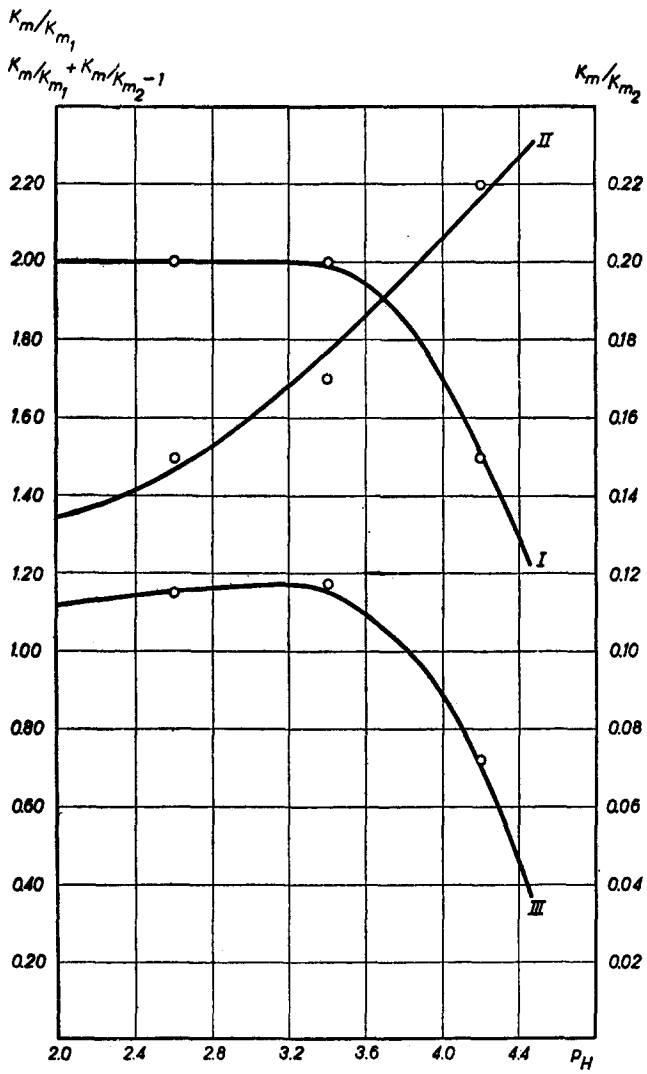

Fig. 2.
I K_m/K_{m_1} II K_m/K_{m_2}
III $K_m/K_{m_1} + K_m/K_{m_2}$-1.

TABLE VI

VALUES OF K_m/K_{m1} K_m/K_{m2} AND CORRECTION TERM AT DIFFERENT p_H

pH	2.0	2.2	2.4	2.6	2.8	3.0	3.2	3.4	3.6	3.8	4.0	4.2	4.4
K_m/K_{m1}	2.0	2.0	2.0	**2.0**	2.0	2.0	2.0	**2.0**	1.9	1.8	1.7	**1.5**	1.3
K_m/K_{m2}	0.14	0.14	0.15	**0.15**	0.15	0.16	0.16	**0.17**	0.18	0.19	0.20	**0.22**	0.24
$K_m/K_{m1} + K_m/K_{m2} - 1$	1.14	1.14	1.15	1.15	1.15	1.16	1.16	1.17	1.08	0.99	0.90	0.72	0.54

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With the correction-terms indicated in Table VI we have calculated the values of k_3 (sal. f.) for all experiments mentioned above. As the values for experiments with citrate-hydrochloric acid buffer do not differ significantly from those for experiments with phosphate-citrate buffer it is to be presumed that the K_{mx}-values in the two different buffer systems are identical or almost identical. K_m is in all calculations put = 0. The results at the 3 p_H-values where experiments are carried out are collected in Table VII.

TABLE VII

VALUES OF k_{obs} AND k_3 AT DIFFERENT p_H

$C_{galactoside}$	$C_{galactose}$	C_{cresol}	$10^4 . k_{obs}/e$ p_H 2.6	k_3 (sal. f.)	$10^4 . k_{obs}/e$ p_H 3.4	k_3 (sal. f.)	$10^4 . k_{obs}/e$ p_H 4.2	k_3 (sal. f.)
0.0400	0.00	0.00	**131**	6.3	**131**	6.3	**134**	6.0
0.0400	0.00	0.00	**135**	6.5	**167**	8.3	**141**	6.5
0.0100	0.00	0.00	480	7.2	553	8.5	503	6.7
0.0200	0.00	0.00	262	6.9	307	8.5	273	6.7
0.0400	0.00	0.00	**125**	5.9	**148**	7.3	**129**	5.8
0.0600	0.00	0.00	75	5.0	95	6.7	82	5.5
0.0800	0.00	0.00	59	5.3	69	6.1	63	5.5
0.1000	0.00	0.00	51	5.3	54	5.9	50	5.2
0.0400	0.00	0.00	**130**	6.2	**149**	7.4	**126**	5.6
0.0400	0.01	0.00	90	5.8	116	7.9	114	6.5
0.0400	0.02	0.00	74	6.2	86	7.4	90	6.5
0.0400	0.04	0.00	51	6.5	71	8.8	62	6.5
0.0400	0.08	0.00	36	6.2	35	7.1	39	6.2
0.0400	0.12	0.00	13	3.1	21	5.9	21	4.6
0.0400	0.00	0.00	**123**	5.6	**151**	7.3	**126**	5.6
0.0400	0.00	0.01	120	5.9	157	7.9	123	5.6
0.0400	0.00	0.02	114	5.6	151	7.9	123	5.9
0.0400	0.00	0.04	114	5.9	147	8.2	114	6.2
0.0400	0.00	0.08	75	4.4	120	7.6	96	5.9
0.0400	0.00	0.12	54	3.4	105	7.0	83	5.6
	average		**129**	5.7	**149**	7.4	**131**	5.9

The mean value of k_3 (sal. f.) at p_H-optimum is $7.4.10^{-4}$. As the value of $10^2.k_3$ for o-cresol-β-d-galactoside with almond emulsin as catalysator is about 6 at p_H 3.43 about 10.4 at p_H-optimum for almond emulsin galactosidase, p_H 4.8, the sal. f.-value of alfalfa seed emulsin, determined with o-cresol-β-d-galactoside as substrate, is 0.074/6 or about 0.012 or 0.074/10.4 or 0.007. These values may be used as comparison values in the investigation of the hydrolysis of other galactosides than o-cresol-β-d-galactoside.

The result of this investigation is, therefore, that with regard to p_H-optimum as well as with regard to K_{mx}-values a significant difference between almond emulsin β-galactosidase and alfalfa seed β-galactosidase seems to exist. One of the differences is that galactose, the affinity of which to almond emulsin β-galactosidase is rather slight ($K_m/K_{m1} \sim 0.26$ at p_H 3.6, $K_m \sim 0.05$), has a very strong affinity to alfalfa seed emulsin β-galactosidase ($K_m/K_{m1} \sim 2$ at p_H 3.4, $K_m \sim 0$). As glucose has about the same affinity to almond emulsin β-galactosidase as galactose ($K_m/K_{m1} \sim 0.12$

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at p_H 4.8, $K_m \sim 0.05$ for galactose, $K_m/K_{m1} \sim 0.13$ at p_H 4.8, $K_m = 0.05$ for glucose), it seems natural to examine the influence of addition of glucose on the velocity of hydrolysis of o-cresol-β-d-galactoside catalysed by alfalfa seed emulsin. Table VIII shows that the K_{m1}-value here is ∞, addition of glucose does not inhibit the hydrolysis of the galactoside at all and no affinity whatever between glucose and alfalfa seed emulsin seems to exist. This is in accordance with the nonhydrolyzability of ß-glucosides by alfalfa seed emulsin.

TABLE VIII

HYDROLYSIS OF O-CRESOL-β-D-GALACTOSIDE WITH ADDITION OF GLUCOSE

Alfalfa seed emulsin. Phosphate-citrate buffer. p_H 3.4. 30°. c = 0.0400 m.

$c_{Glucose}$	$k'.10^4/e$	10^4k_3 (sal. f.)	10^4k_3 (sal. f.) calculated with $Km/Km_1 \sim 0.25$ as for almond emulsin galactosidase.
0.00	145	7.0	7.0
0.01	150	7.3	7.6
0.02	149	7.6	8.6
0.04	147	6.7	8.0
0.08	146	6.4	9.2
0.12	132	6.4	10.5
	average	6.9	
	previously found	7.4	

In order to examine if an exchange of prostetic groups between almond emulsin and alfalfa seed emulsin can possibly take place the following experiment was made:

To 0.0400 m solutions of o-cresol-β-d-glucoside and o-cresol-β-d-galactoside were added at p_H 3.6 (about the p_H-optimum of alfalfa seed emulsin) and at p_H 4.4 (about the p_H-optimum of almond emulsin) mixtures of the two enzyme-solutions with known amounts of each of the enzymes. From the previously determined values of k'_{obs}/e for these glycosides and glycosidases was calculated the k'_{obs}-value to be expected if the action of the two glycosidases is additional and the two enzymes consequently do not exchange their prostetic groups. The Tables IX-XII give the results of the experiments.

TABLE IX

HYDROLYSIS OF O-CRESOL-β-D-GLUCOSIDE

p_H 3.6. Phosphate-citrate buffer. 30°. $c_{Glucoside} = 0.0400$. $e_{Almond} = 0.0028$, $e_{Alfalfa} = 0.0323$. $k'/e_{Almond} = 38.10^{-2}$, $k'/e_{Alfalfa} = 0$. $10^2.k'_{calc.} = 38 \cdot 0.0028 = 0.106$.

t min	x	$10^4.k'$	$x_{calc.}$
90	0.26	6.90	0.38
180	0.50	7.15	0.69
270	0.61	6.03	0.94
360	0.79	6.26	1.14
∞	1.95	—	1.95
		average 6.6	

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TABLE X

HYDROLYSIS OF O-CRESOL-β-D-GLUCOSIDE

pH 4.4. Phosphate-citrate buffer. 30°. $c_{Glucoside} = 0.0400$. $e_{Almond} = 0.0014$, $e_{Alfalfa} = 0.0323$. $k'/e_{Almond} = 57.10^{-2}$, $k'/e_{Alfalfa} = 0$. $10^2.k'_{calc.} = 57 \cdot 0.0014 = 0.08$.

t min	x	$10^4.k'$	$x_{calc.}$
90	0.30	8.06	0.30
180	0.56	8.17	0.55
270	0.80	8.49	0.76
360	0.96	8.18	0.95
∞	1.95	—	1.95
		average 8.2	

TABLE XI

HYDROLYSIS OF O-CRESOL-β-D-GALACTOSIDE

pH 3.6. Phosphate-citrate buffer. 30°. $c_{Galactoside} = 0.0400$, $e_{Almond} = 0.0111$, $e_{Alfalfa} = 0.0323$ $k'/e_{Alm.} = 5.3.10^{-2}$, $k'/e_{Alf.} = 1.6.10^{-2}$. $10^2k'_{calc.} = 5.3 \cdot 0.0111 + 1.6.0.0323 = 0.102$

t min	x	$10^3.k'$	$x_{calc.}$
90	0.33	10.28	0.17 + 0.18 = 0.35
180	0.55	9.29	0.32 + 0.33 = 0.65
270	0.69	8.25	0.46 + 0.48 = 0.94
360	0.84	8.08	0.58 + 0.60 = 1.18
∞	1.72	—	1.72
		average 9.0	

TABLE XII

HYDROLYSIS OF O-CRESOL-ß-D-GALACTOSIDE

pH 4.4. Phosphate-citrate buffer. 30°. $c_{Galactoside} = 0.0400$. $e_{Almond} = 0.0083$, $e_{Alfalfa} = 0.0323$ $k'/e_{Alm.} = 8.3.10^{-2}$, $k'/e_{Alf.} = 1.2.10\text{-}2$. $10^2.k_{calc.} = 8.3 \cdot 0.0083 + 1.2 \cdot 0.0323 = 0.108$

min t	x	$10^4.k'$	$x_{calc.}$
90	0.30	9.25	0.23 + 0.13 = 0.36
180	0.56	9.50	0.43 + 0.26 = 0.69
270	0.72	8.72	0.60 + 0.37 = 0.97
360	0.86	8.36	0.75 + 0.48 = 1.23
∞	1.72	—	1.72
		average 9.0	

At a p_H-value unfavourable for the β-glucosidase (but optimum for alfalfa seed galactosidase) alfalfa seed emulsin acts as a somewhat inhibiting impurity. At the p_H-optimum of the β-glucosidase alfalfa seed emulsin has no action whatever.

Against the β-galactoside both almond emulsin and alfalfa seed emulsin acts normally as β-galactosidases. The combined action is, though, not fully that calculated as the sum of the action of the two β-galactosidases.

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In the experiments described here, the mixture of the two enzymes took place immediately before the experiment was started. In other experiments the mixture of the two enzymes was allowed to stand 12 hours before adding it to the substrate solution. The results were practically the same as those described.

Summarizing the results of these experiments it may be said that they stress the significance of the colloidal carrier which is to be supposed as combined with the prostetic group of the enzyme.

If the necessity of assuming quite a series of different β-galactosidases, one for each source of enzyme (the very closely related, e.g., different members of the *prunace*-family excepted) is not to be inevitable, it must be concluded from the experiments that both the p_H-optimum of the enzyme and the affinity between enzyme and substrate is determined principally by the colloidal carrier, to such a degree that some glycosides are completely unaffected by enzyme preparations which from their faculty of catalysing the hydrolysis of related glycosides might be expected to contain the β-glycosidase in question.

As long as the reversible dissociation of the colloidal carrier and the prostetic group has not been experimentally verified it seems useless to discuss the identity or non-identity of β-glucosidase and β-galactosidase, if thereby is considered the identity of their prostetic groups. If, on the contrary, the total enzyme complex is considered, it must be concluded from this and the preceding papers (VEIBEL, MØLLER, AND WANGEL [6], VEIBEL, WANGEL, AND ØSTRUP[5]) that at least 2 different enzymes, a β-glucosidase and a β-galactosidase, exist, but that it seems obvious that each of these two types of enzymes comprises more species so different that they are to be regarded as individual enzymes.

SUMMARY

The β-galactosidase of alfalfa seed emulsin is, as first found by HILL [1], unable to catalyse the hydrolysis of β-glucosides and must, consequently, be regarded as different from β-glucosidase. A closer study has shown that it differs from the β-galactosidase of almond emulsin with regard to the p_H-optimum and affinity to β-galactosides, galactose and aglucone, e.g., o-cresol.

It is discussed if differences between two β-glycosidases means differences between their prostetic groups or between the colloidal carriers of these groups.

The opinion is expressed that no definite answer to this question can be given until methods of producing the reversible dissociation of the enzymes into prostetic groups and colloidal carriers have been found.

RÉSUMÉ

La β-galactosidase de l'émulsine des graines d'alfalfa trouvée d'abord par HILL [1], est incapable de catalyser l'hydrolyse des β-glucosides et doit, par conséquent, être considérée comme étant différente de la β-glucosidase. Une étude plus précise a montré qu'elle diffère de la β-galactosidase de l'émulsine d'amandes relativement à l'optimum du p_H et à l'affinité pour les β-galactosides, le galactose et l'aglucone, p. ex. o-crésol.

La discussion tend à déterminer si des différences existant entre deux β-glycosidases indiquent des différences entre leurs groupes prosthétiques ou entre les phérons colloïdaux de ces groupes.

L'opinion est émise qu'aucune réponse précise ne pourra être donnée à cette question avant que l'on ait trouvé des méthodes pouvant produire la dissociation réversible des enzymes en groupes prosthétiques et en phérons colloïdaux.

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ZUSAMMENFASSUNG

Die β-Galaktosidase des Luzerne-emulsins ist, wie zuerst von HILL [3] gefunden, unfähig die Hydrolyse von β-Glukosiden zu katalysieren und muss infolgedessen als verschieden von der β-Glukosidase betrachtet werden.

Eine nähere Untersuchung zeigte, dass sie sich von der β-Galaktosidase des Mandelemulsins in Bezug auf das p_H-Optimum und auf die Affinität zu β-Galaktosiden, Galaktose und Aglukon, z.B. *o*-Kresol, unterscheidet.

Es wird diskutiert, ob Unterschiede zwischen zwei β-Glukosidasen Unterschiede zwischen deren prosthetischen Gruppen oder zwischen den kolloidalen Trägern dieser Gruppen bedeuten.

Die Verfasser sind der Meinung, dass keine bestimmte Antwort auf diese Frage gegeben werden kann, bevor Methoden ausgearbeitet sind, welche eine umkehrbare Dissoziation der Enzyme in prosthetische Gruppen und kolloidale Träger ermöglichen.

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