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Faecal Chymotrypsin – A New Photometric Method Using N-Acetyl-*L*-tyrosine Ethyl Ester as Substrate[1])

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Summary: A photometric method for chymotrypsin is proposed, using the substrate N-acetyl-*L*-tyrosine ethyl ester, which is already used in the titrimetric procedure. Hydrolysis of the ester bond produces equal amounts of ethanol and acetyltyrosine, the latter being measured in the titrimetric method. The ethanol can easily be measured by the alcohol dehydrogenase method in the trichloroacetic acid supernatant. Suitable test conditions are reported.

Chymotrypsin im Stuhl – eine neue photometrische Methode mit dem Substrat Acetyltyrosylethylester

Zusammenfassung: Es wird vorgeschlagen, das im titrimetrischen Verfahren bewährte Substrat N-Acetyl-*L*-tyrosylethylester für eine photometrische Methode zu verwenden. Nach hydrolytischer Spaltung der Esterbindung wird neben der titrierbaren Säure Acetyltyrosin äquimolar Ethanol frei. Dieses kann einfach im klaren Trichloressigsäureüberstand enzymatisch gemessen werden. Die geeigneten Meßbedingungen werden beschrieben.

Introduction

The determination of faecal chymotrypsin is generally accepted as the first step in evaluating the exocrine pancreatic function. Until now, faecal chymotrypsin activity has been measured reliably only by following titrimetrically the hydrolysis of N-acetyl-*L*-tyrosine ethyl ester in a pH-stat (2).

Several different photometric procedures, using amide substrates have been suggested for use in less specially equipped routine laboratories. Since the enzyme is bound to stool particles in varying amounts from 50 to almost 100% (3), methods using the clear faecal supernatant (4) are not applicable. The substrate must be incubated with uncentrifuged stool suspension before the coupling reaction can be carried out in the clear supernatant after acid precipitation and centrifugation (5). The results of these methods, however, do not correlate well with the results of the titrimetric test. Photometric procedures give consistently lower faecal chymotrypsin concentrations even if carried out with pure bovine chymotrypsin as standard for calibration. According to our current results using the amide substrate N-benzoyl-*L*-tyrosyl-*p*-aminobenzoic acid (6), part of the reaction product *p*-aminobenzoic acid remained bound to faecal particles, resulting in lower concentrations after centrifugation.

This would offer a possible explanation for the bad correlation between titrimetric and photometric methods found with faecal material and the good agreement between these two methods when assaying duodenal fluid (7). We therefore developped a photometric test that makes use of the substrate N-acetyl-*L*-tyrosine ethyl ester (already used in the titrimetric procedure), assuming that "particle binding" would not occur for the small molecule ethanol.

Materials and Methods

All reagents were p.a. grade from Merck Darmstadt (FRG).

Buffer: Tris 0.15 mol/l, NaCl 0.5 mol/l, $CaCl_2$ 0.08 mol/l, adjusted to pH 9.0 with HCl.

Substrate solution: 5 g N-acetyl-*L*-tyrosine ethyl ester (ATEE, Merck 83) are dissolved in 100 ml dimethylsulfoxide (DMSO, Merck 2950).

Precipitant: Trichloroacetic acid 3.3 mol/l.

[1]) Preliminary results have been reported at the Joint Congress of the Scandinavian and German Societies of Clinical Chemistry in Hamburg, October 8–11, 1980 (1).

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Chymotrypsin standard: Crystallized, lyophilized bovine chymotrypsin (Merck 2307). A stock solution of 1 g/l 0.15 mol/l NaCl is prepared and frozen in small portions. Before use it is further diluted 200 fold to give a final concentration of 5 mg/l.

Ethanol test: Alcohol dehydrogenase method (8). Reagents e.g. Testcombination "Blood alcohol" (Boehringer Mannheim 123 960).

Stool samples of about 5 g are collected on three different days and stored refrigerated up to 1 week at 8–4 °C until analysis.

Results and Discussion

Ethanol as a parameter of the enzymatic hydrolysis of N-acetyl-*L*-tyrosine ethyl ester

Chymotrypsin hydrolyses 1 molecule of N-acetyl-*L*-tyrosine ethyl ester to give 1 molecule of N-acetyl-tyrosine and 1 molecule of ethanol. Figure 1 shows that under suitable experimental conditions the titratable acid equivalent and free alcohol agree with the theoretical stoichiometric relationship of 1:1. Faecal material and pure bovine chymotrypsin give similar results (for details see legend to fig. 1).

While the accumulation of N-acetyltyrosine can be determined continuously by alkali titration, the amount of released ethanol can only be measured as the final concentration. In a continuous photometric test the requirement for sufficient faecal (particle) concentration and continuous agitation cannot be satisfied.

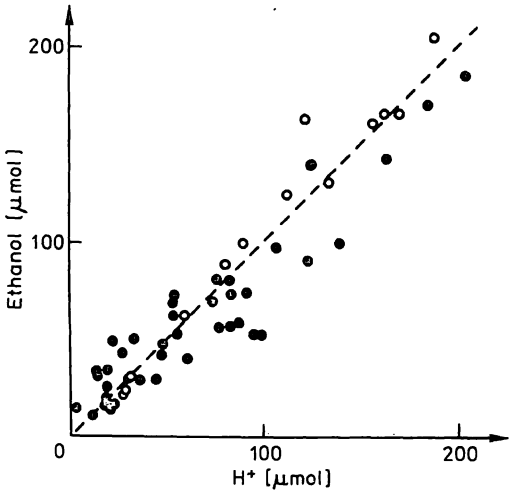

Fig. 1. Equimolar production of ethanol and H^+ following hydrolysis of N-acetyl-*L*-tyrosine ethyl ester using faecal suspension (•) or crystalline bovine chymotrypsin (○).

Reaction mixture: 50 mmol/l tris buffer pH 9.0 containing 50 mmol/l $CaCl_2$, 500 mmol/l NaCl, 37 mmol/l N-acetyl-*L*-tyrosine ethyl ester and 200 ml/l dimethylsulfoxide. Total volume 25 ml.

Incubation in sealed tubes at 25 °C with constant agitation. After 15 minutes an aliquot was precipitated with trichloroacetic acid (final concentration 0.3 mol/l) for ethanol determination by the alcohol dehydrogenase method. H^+ liberated was assayed by immediate back titration of the incubation mixture to pH 9.0 using 10 mmol/l NaOH.

Regression line: $y = 1.01\ x - 2$, $r = 0.90$, $n = 40$ faecal samples + 14 chymotrypsin standards.

Establishing optimal conditions

Choice of solvent for N-acetyl-L-tyrosine ethyl ester

N-acetyl-*L*-tyrosine ethyl ester is not water soluble. The usual solvent, methanol, cannot be used since alcohol dehydrogenase is not sufficiently ethanol-specific. N-acetyl-*L*-tyrosine ethyl ester is, however, very soluble in dimethylsulfoxide. In the conventional titrimetric assay, dimethylsulfoxide, at our chosen final concentration of 200 ml/l, leads to 60–70% enhancement of both faecal and pure bovine chymotrypsin activity (fig. 2). Since the faecal enzyme concentration is calibrated using bovine chymotrypsin, this stimulation by dimethylsulfoxide has no influence on the final result.

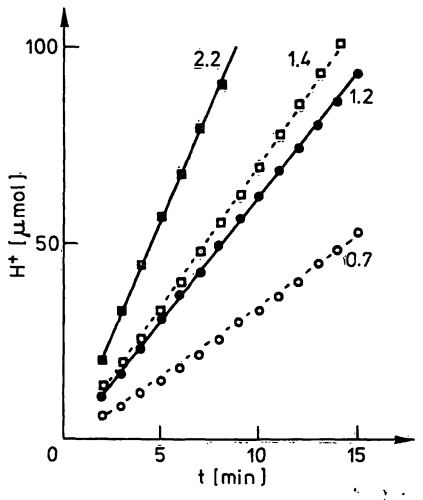

Fig. 2. pH-stat titration of faecal (○●) and bovine chymotrypsin (□■), with methanol (open symbols) or dimethylsulfoxide (solid symbols) as the solvent. N-acetyl-*L*-tyrosine ethyl ester concentration constant at 20 mmol/l. Other reaction conditions as with the standard titrimetric procedure. The numbers indicate the slopes of the various titration curves.

Spontaneous hydrolysis of N-acetyl-L-tyrosine ethyl ester

During the incubation at pH 9 there is virtually no spontaneous hydrolysis of the substrate, however, once in trichloroacetic acid, there is a time- and concentration-dependent release of ethanol (fig. 3). In perchloric acid, this effect is even more evident. To give an adequate inactivation and precipitation of the test reagents the minimum concentration of trichloroacetic acid that could be used was 0.3 mol/l. The inclusion of a blank control tube in the assay allowed the measured enzyme activities to be corrected for the accompanying spontaneous hydrolysis. In routine conditions this correction was always less than 10% of the total activity, and can be further reduced by carrying out the assay in an ice bath.

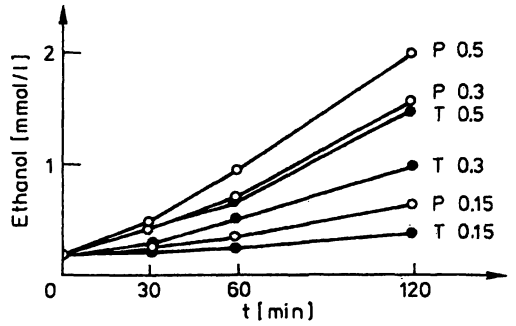

Fig. 3. Spontaneous hydrolysis of N-acetyl-*L*-tyrosine ethyl ester with various concentrations (mol/l) of perchloric acid (P) or trichloroacetic acid (T) at room temperature.

Substrate concentration and pH

Although an N-acetyl-*L*-tyrosine ethyl ester concentration of 14 mmol/l is used in the titrimetric procedure, we found that even at 50 mmol/l saturation was still not attained. As a compromise between optimal substrate concentration and economy we chose the arbitrary concentration of 37 mmol/l (fig. 4).

There is a broad pH-optimum around pH 9.0 (fig. 5).

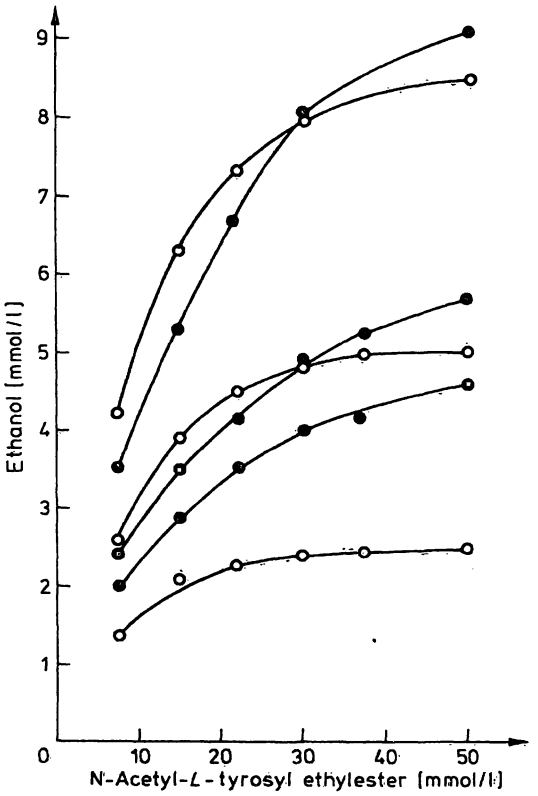

Fig. 4. Ethanol production from N-acetyl-*L*-tyrosine ethyl ester as a function of substrate concentration. —o— bovine chymotrypsin, —•— faecal suspension. With the exception of varying N-acetyl-*L*-tyrosine ethyl ester concentrations the test procedure was acc. to our "final test procedure".

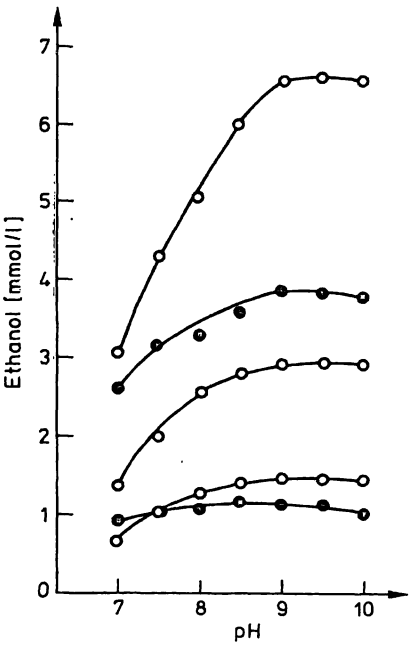

Fig. 5. pH dependence of the enzymatic hydrolysis of N-acetyl-*L*-tyrosine ethyl ester. —o— bovine chymotrypsin, —•— faecal suspension. The test procedure was acc. to our "final test procedure" using tris buffer with varying pH.

Final test procedure

Suspend approximately 1 g faeces (accurately weighed for final correction) in 20 ml of saline (0.15 mol/l NaCl) and allow to stand overnight in a refrigerator in closed tubes.

Mix thoroughly (Whirlmix). Dilute 1 ml faecal suspension with 4 ml saline to give a final concentration of faeces of about 10 mg/ml. Use large bore pipette tips for all sampling of stool suspension (for example cut a Marburg pipette tip by 4 mm before use). Prepare reagents acc. to "materials".

Mix 1 ml of diluted faecal suspension (as well as 1 ml chymotrypsin standard dilution (5 mg/l) and 1 ml saline blank respectively) with 3 ml buffer solution[2]). Start the reaction by the addition of 1 ml substrate solution. All tests are run in duplicate. Seal the tubes and incubate 15 minutes at 25 °C with continual gentle shaking. Stop the reaction by addition of 0.5 ml precipitant. Centrifuge at 2000 *g* for 10 minutes. For immediate determination of the ethanol concentration mix 25 µl of the supernatant with 1 ml alcohol reagent mixture prepared according to the manufacturer's instructions. Results are given as chymotrypsin per wet weight of faeces (µg/g). The calculation is as follows:

$$\text{Chymotrypsin [µg/g faeces]} = \frac{A_{\text{Sample}}}{A_{\text{Standard}}} \times 5 \times 100$$

5 = Concentration of standard (mg/l)

100 = Dilution of faeces

[2]) The total volume of the reaction mixture should not be further reduced, since addition of too small a quantity of faeces would not provide a representative proportion of chymotrypsin-active particles.

All results are corrected for the exact weight of the stool portion assayed.

Reference values: As with the titrimetric procedure values below 120 μg/g indicate probability of pancreatic deficiency.

Linearity and precision

The method as described shows good linearity as a function of incubation time (fig. 6) as well as of faecal or chymotrypsin concentration (fig. 7).

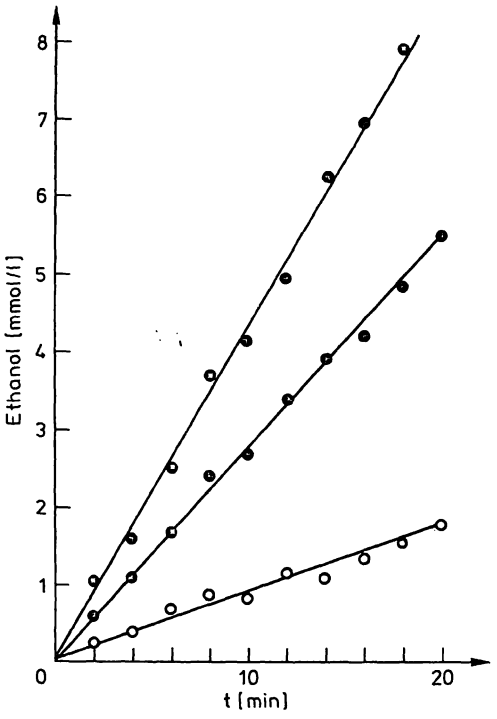

Fig. 6. Time course of ethanol production from N-acetyl-*L*-tyrosine ethyl ester by faecal suspension (•) or bovine chymotrypsin (o) under our standard conditions.

The day to day coefficient of variation for three different stools was as follows:

Stool	$\bar{x}$ (μg/g)	CV (%)	N
I	68	5.0	10
II	294	5.5	10
III	742	4.6	10

The within-day precision calculated from 150 routinely performed duplicate assays was 11%, the activities ranging from 20 to 2000 μg/g.

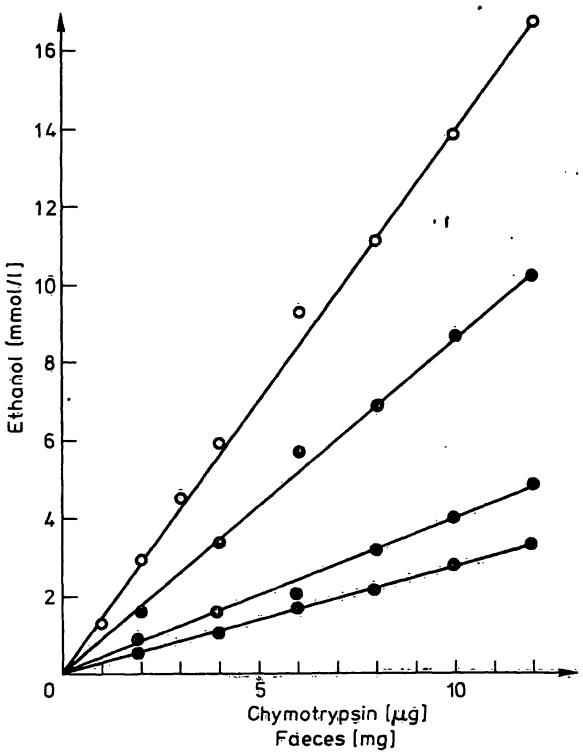

Fig. 7. Effect of the amount of enzyme added per test on ethanol production from N-acetyl-*L*-tyrosine ethyl ester by faecal suspension (•) or bovine chymotrypsin (o). Incubation for 15 minutes at 25 °C.

Conclusion

Our photometric method makes use of the well established substrate N-acetyl-*L*-tyrosine ethyl ester, as well as uncentrifuged faecal suspension. The reaction product, ethanol, can be measured easily in the clear trichloroacetic acid supernatant without any loss in recovery. Therefore the activity determined in faecal suspension compared to bovine chymotrypsin as a standard is similar to that obtained by the titration method. All previous clinical experience with regard to sensitivity, specificity and reference region can be used.

The test is linear over a wide range of activities. Large series of determinations can be made relatively quickly without special laboratory equipment. The procedure in our hands has successfully proved itself over two years.

Acknowledgement

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