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New Effective Method for Analysis of the Component Composition of Enzyme Complexes from *Trichoderma reesei*

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Abstract—A method for analysis of the component composition of multienzyme complexes secreted by the filamentous fungus *Trichoderma reesei* was developed. The method is based on chromatofocusing followed by further identification of protein fractions according to their substrate specificity and molecular characteristics of the proteins. The method allows identifying practically all known cellulases and hemicellulases of *T. reesei*: endoglucanase I (EG I), EG II, EG III, cellobiohydrolase I (CBH I), CBH II, xylanase I (XYL I), XYL II, β-xylosidase, α-L-arabinofuranosidase, acetyl xylan esterase, mannanase, α-galactosidase, xyloglucanase, polygalacturonase, and exo-β-1,3-glucosidase. The component composition of several laboratory and commercial *T. reesei* preparations was studied and the content of the individual enzymes in these preparations was quantified. The influence of fermentation conditions on the component composition of secreted enzyme complexes was revealed. The characteristic features of enzyme preparations obtained in "cellulase" and "xylanase" fermentation conditions are shown.

Key words: *Trichoderma reesei*, chromatofocusing, component composition, cellulases, hemicellulases, fermentation

The microscopic fungus *Trichoderma reesei* is one of the most widely used industrial microorganisms producing enzymes of the cellulase and hemicellulase complex. High level of secretion and diversity of the produced enzymes with various substrate specificities promotes wide application of enzyme preparations from *T. reesei* in different fields of biotechnology: in bioconversion of renewable plant raw stock and converting it to fuel and other useful products; in food industry, in the process of wine production, brewing, production of extractive substances; as an additive to farm livestock fodder; in textile industry, for improving quality of cotton and linen articles; in biobleaching of cellulose pulp in paper production, etc. [1-6].

Application of cellulases and hemicellulases from *T. reesei* in various biotechnological processes is associated with the need for different (often fairly distinct) types of substrate conversion. In turn, this imposes different requirements on the composition of *T. reesei* enzyme complexes as well as properties of individual enzymes in the complex. Hence, different application areas require production of enzyme complexes with various (however, completely determined) component composition. Therefore, genetic engineering methods are used (for instance, increased expression of certain enzymes and decreased expression of others [7-12]); different mutant *T. reesei* strains are obtained; fermentation conditions are varied [13, 14], etc. Obtaining strains that produce a certain set of enzymes requires continuous monitoring of the component composition of the produced enzyme complexes. Therefore, the development of an efficient and rapid technique for analysis of component composition of *T. reesei* enzyme complexes is a high priority task. Two-dimensional gel electrophoresis and preparative capillary isoelectrofocusing are now most often used for determination of component composition of multienzyme *T. reesei* samples [15, 16]. The mentioned techniques have a number of disadvantages, among which one can mention difficulties in identification of individual components in the complex, long time of analysis, and the need for special equipment.

The goal of this work was to develop a simple, quick, and efficient analysis technique for determination of component composition of exocellular enzyme complex-

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es from *T. reesei* based on preparative chromatofocusing using a chromatographic FPLC system.

MATERIALS AND METHODS

Enzyme preparations. The following laboratory preparations of *T. reesei* were used in this work: SA No. 210.29 and C-XL No. 592.2, which were produced at the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino). Both preparations were ultraconcentrates of culture broth of *T. reesei* A1 strain cultivated under different fermentation conditions. In this work, we also used a dry industrial sample of Celloviridin G20X from the Berdsk factory for biological preparations (Russia) and liquid sample of Celloviridin G2X from JS Belmedpreparaty (Belarus); both enzyme preparations are produced using *T. reesei* 18.2KK strain.

Substrates. Sodium salt of carboxymethyl cellulose (CMC) of medium viscosity, glucuronoxylan from birch, potassium salt of polygalacturonic acid (PGU), *p*-nitrophenyl-β-D-lactopyranoside (PNP-β-Lac), *p*-nitrophenyl-β-D-glucopyranoside (PNP-β-Glu), *p*-nitrophenyl-β-D-cellobioside (PNP-β-Cell), *p*-nitrophenyl-α-D-galactopyranoside (PNP-α-Gal), *p*-nitrophenyl-α-L-arabinofuranoside (PNP-α-L-Af), *p*-nitrophenyl-β-D-xylopyranoside (PNP-β-Xyl), and *p*-nitrophenyl-acetate were purchased from Sigma (USA). β-Glucan from barley and xyloglucan from tamarind were from Megazyme (Australia). Chromatographic filter paper No. 1 was from Whatman (UK), microcrystalline cellulose (MCC) avicel was from Serva (Germany). Partially depolymerized galactomannan from acacia was kindly provided by V. D. Scherbukhin (Institute of Biochemistry, Russian Academy of Sciences).

Separation of *T. reesei* enzyme complexes into individual components included two main steps: preliminary purification from non-protein contaminants (insoluble substances, carbohydrates, and coloring agents) and fine fractioning. During the preliminary purification, the samples were salted out using ammonium sulfate (80% saturation at 0°C). The residue formed after 12 h (at 4°C) was centrifuged for 30 min at 11,000 rpm and 4°C. Desalting was performed on an Econo System chromatographic system (Bio-Rad, USA) using a column with Biogel P-4 carrier (gel volume 50 ml) equilibrated with 25 mM imidazole-HCl buffer, pH 7.5, 1 ml/min flow rate.

The fine fractioning procedure involved preparative chromatofocusing of the desalted samples. Chromatofocusing was performed on an FPLC liquid chromatograph (Pharmacia-Biotech, Sweden) on a Mono P HR 5/20 column (Pharmacia-Biotech; gel volume 4 ml) equilibrated with initial buffer (25 mM imidazole-HCl, pH 7.5). Proteins bound under the initial conditions were eluted by a pH gradient maintained by sequential injections of PB 74 Polybuffer (Pharmacia-Biotech) with pH 4.0 and 3.0 into the column; flow rate was 0.7 ml/min.

Determination of enzyme activities. CMCase, β-glucanase, avicelase, xylanase, mannanase, polygalacturonase, and xyloglucanase activities were determined according to initial formation rate of the reducing sugars (RS) using Somogyi–Nelson technique for the hydrolysis of CMC, β-glucan, avicel, xylan, galactomannan, PGU, and xyloglucan, respectively [17, 18]. In general, one activity unit for hydrolysis of polysaccharides corresponded to the enzyme amount required for the formation of 1 μmol of RS in 1 min from a certain substrate at 50°C and pH 5.0. Standard substrate concentration in reaction mixture in the case of polysaccharide substrates was 5 g/liter. The activity towards filter paper (FPA) was determined according to a standard technique [17], where RS were detected using the dinitrosalicylic technique.

Enzyme activities towards low molecular weight synthetic substrates (*p*-nitrophenyl-glycosides PNP-β-Lac, PNP-β-Glu, PNP-β-Cell, PNP-α-Gal, PNP-α-L-Af, and PNP-β-Xyl) were determined based on initial rate of *p*-nitrophenol (PNP) formation [17, 18]. The solution (0.5 mM) of PNP-glycoside substrate in 0.1 M sodium acetate buffer, pH 5.0, was incubated with enzyme at 40°C for 10 min. The reaction was terminated by adding 1 M Na_2CO_3 solution. After 10 min, PNP formed in the solution was determined spectrophotometrically at 400 nm using molar absorption coefficient ε_{400} = 18,300 $M^{-1}\cdot cm^{-1}$. Activity towards PNP-acetate was determined based on initial rate of PNP formation according to a modified technique [17]. The technique was modified due to the instability of substrate at alkaline pH values. The solution (0.5 mM) of PNP-acetate in 0.1 M Na-acetate buffer, pH 5.0, was incubated with enzyme at 40°C for 10 min. Then 1 M Tris-HCl buffer (pH 7.5) was added to the reaction mixture; immediately afterward, *p*-nitrophenol formed in the solution was determined spectrophotometrically at 400 nm using molar absorption coefficient ε_{400} = 9200 $M^{-1}\cdot cm^{-1}$. Activities upon PNP-glycosides and PNP-acetate were expressed in international units (1 activity unit corresponds to the formation of 1 μmol of product per 1 min under the action of enzymes towards the corresponding substrate).

Determination of biochemical characteristics of individual enzymes. For electrophoresis in polyacrylamide gel (gel size 70 × 80 × 0.75 mm) under denaturing conditions (SDS-PAGE) with concentrating (4%) and separating (12%) gels and also for isoelectrofocusing (IEF) in 4% polyacrylamide gel (125 × 65 × 0.75 mm) the reagents and kits from Reanal (Hungary) and Sigma and Bio-Rad (USA) were used. Analytical isoelectrofocusing of individual proteins was performed on a Model 111 Cell apparatus (Bio-Rad); SDS-PAGE was performed on a Mini

Protean II (Bio-Rad) apparatus according to the operation manual. Proteins in gels were stained with Coomassie R-250 from Ferak (Germany). Before electrophoresis, the investigated enzyme solutions were initially treated with 1% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol at 100°C for 5-10 min. As standards for SDS-PAGE and IEF, we used protein marker mixtures from Sigma.

RESULTS AND DISCUSSION

Development of express analysis of component composition of *T. reesei* enzyme complexes. The proposed analysis technique for component composition of *T. reesei* enzyme complexes included the following steps: (i) non-protein contaminants were removed from the preparations, and (ii) the preparations were subjected to fine fractioning using chromatofocusing over a wide pH range (from 7.5 to 3.0). Fractions obtained after the second step (most of them contained virtually homogeneous proteins) were subjected to SDS-PAGE and IEF; their activities toward a wide range of natural and synthetic substrates were also determined. The comparison of biochemical characteristics (M_r and p*I*) and the substrate specificity of purified fractions (enzymes) with analogous properties of *T. reesei* enzymes described in the literature [19-22] allowed us to identify the individual enzymes in fractions (in chromatographic peaks) and perform quantitative estimation of their content in the investigated enzyme preparations.

The initial enzyme preparation employed in the development of the technique was laboratory preparation *T. reesei* SA No. 210.29. After preliminary purification, it was subjected to fine purification via chromatofocusing. To create a gradient we used a system of three buffers: starting buffer 25 mM imidazole-HCl, pH 7.5, and eluting buffers Polybuffer 74, pH 4.0 and 3.0. Totally 30 protein fractions were collected during chromatofocusing. For the analysis of substrate specificity of the fractions, we used 14 high and low molecular weight substrates of cellulases, hemicellulases, and pectinases (see "Materials and Methods"). Fractions, which had the same pattern of activities, were pooled. As a result, we obtained 17 pooled protein fractions (PF) (Fig. 1). Based on substrate specificity of the PF, data from SDS-PAGE and IEF of enzymes (data for certain enzymes are presented in Figs. 2 and 3), and also present literature data about molecular characteristics of *T. reesei* enzymes [19-22] (summarized in http://afmb.cnrs-mrs.fr/CAZY) we could make conclusions about component composition of the SA No. 210.29 preparation.

Identification of enzymes in the PF can be illustrated with specific examples. PF 6 had high activities towards CMC, xylan, PNP-β-Lac, and PNP-β-Cell. Literature data [19, 22] reveal that the only *T. reesei* enzyme which exhibits such substrate specificity is endoglucanase I (EG I). Also, molecular characteristics

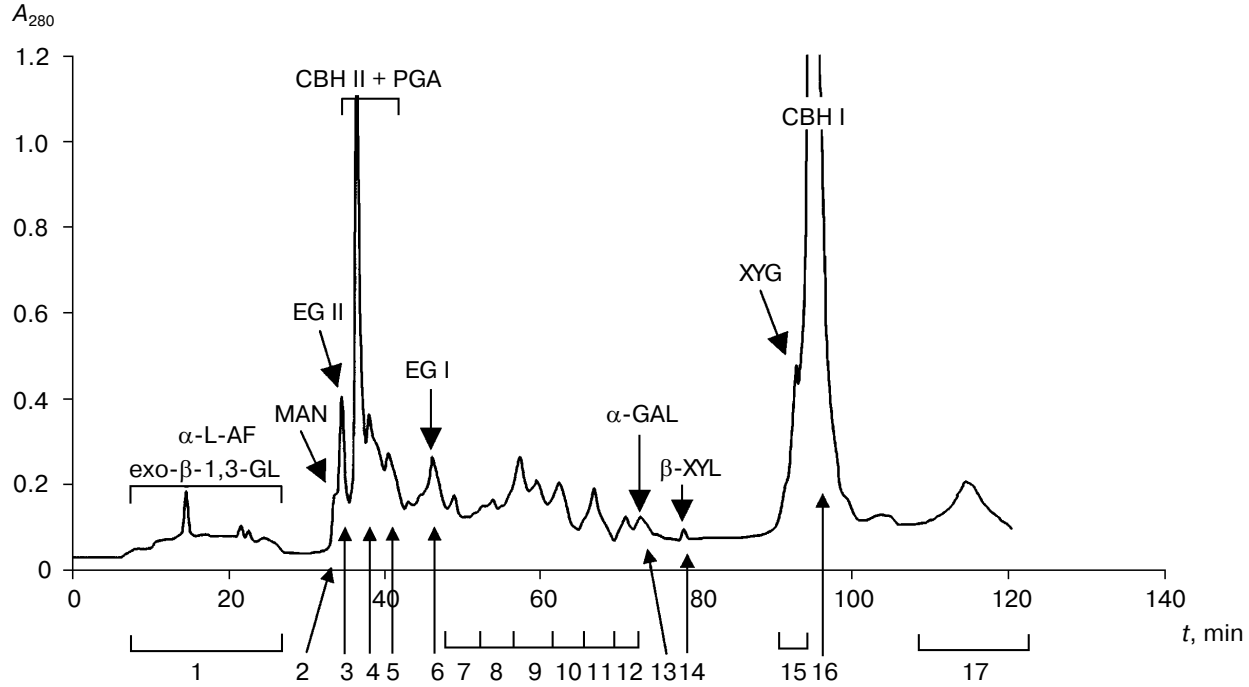

Fig. 1. Chromatofocusing profile for *T. reesei* SA No. 210.29 preparation.

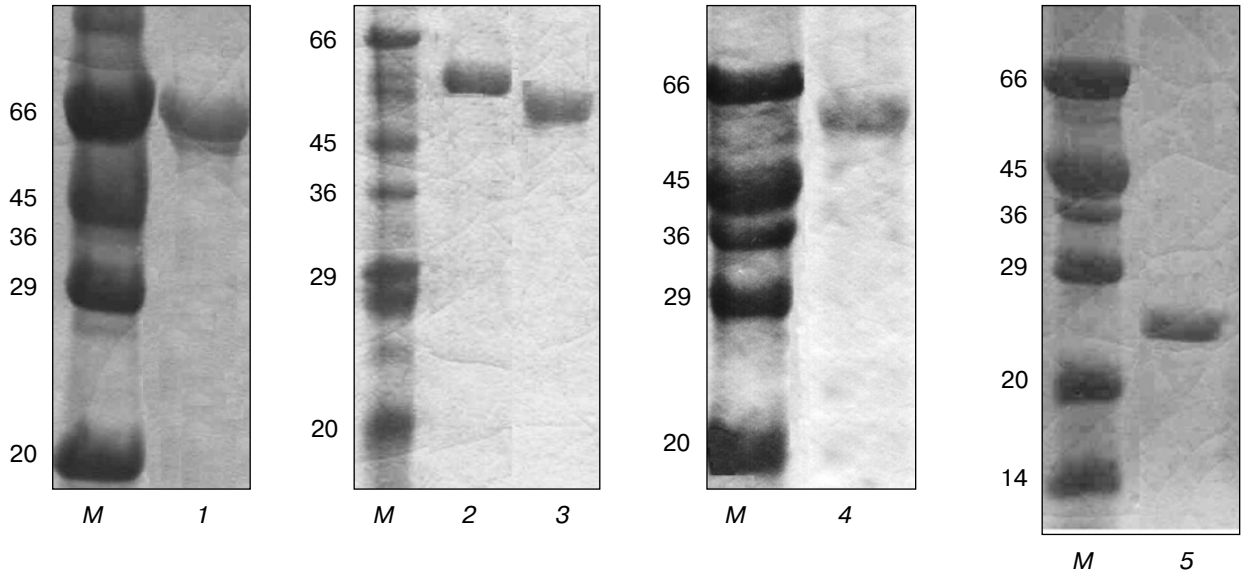

Fig. 2. SDS-PAGE of purified enzymes: *1*) CBH I; *2*) CBH II; *3*) EG II; *4*) EG I; *5*) EG III. *M*, protein markers with different molecular weights (in kD).

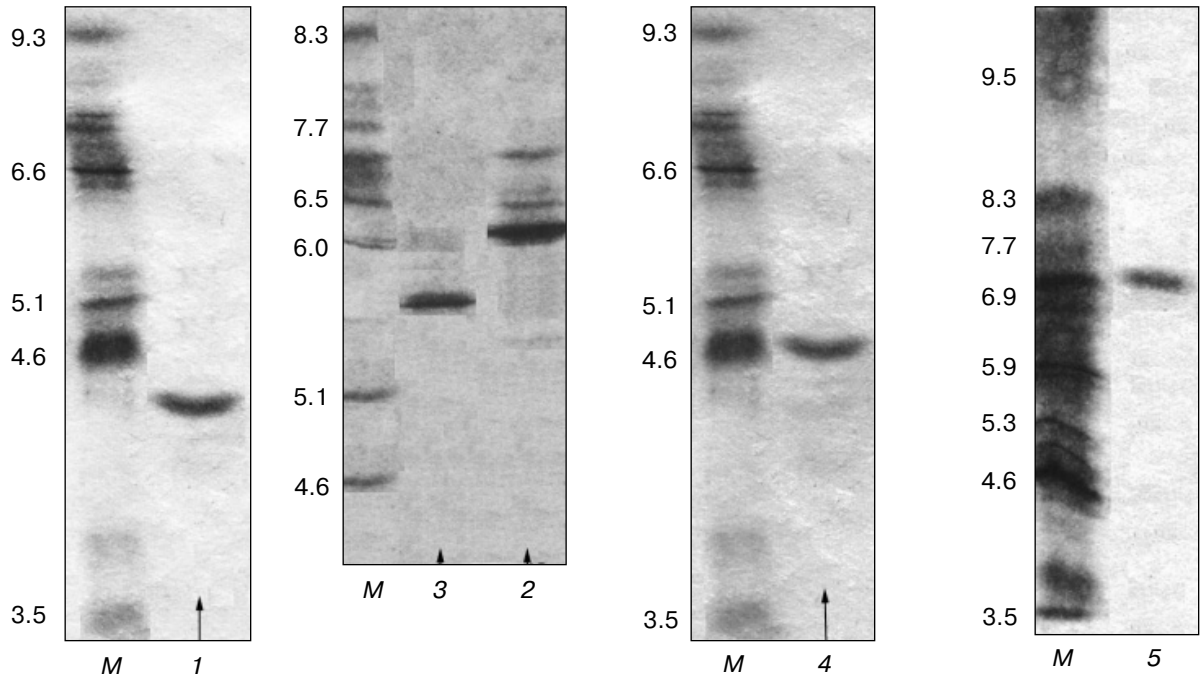

Fig. 3. IEF of purified enzymes: *1*) CBH I; *2*) CBH II; *3*) EG II; *4*) EG I; *5*) EG III. *M*, protein markers with different p*I* values.

of the enzyme correlate well with the literature data (molecular weight of enzyme contained in this fraction was 57 kD, p*I* 4.6). PF 16 had high avicelase activity and activity towards PNP-β-Lac and PNP-β-Cell; at the same time, no CMCase or any other activities were detected. The mentioned activity pattern is characteristic for cellobiohydrolases; moreover, among two *T. reesei* cellobiohydrolases known from the literature [19-21], only cellobiohydrolase I (CBH I) has not only high avicelase activity but also activity towards such low molecular

Table 1. Component composition of pooled *T. reesei* SA No. 210.29 fractions

Fraction	Component composition	Substrate specificity
PF 1	Mixture of α-L-AF and exo-β-1,3-GL	PNP-α-L-Af and PNP-β-Glu
PF 2	MAN	Galactomannan
PF 3	EG II	CMC, β-glucan
PF 4-5	CBH II with low content of PGA	Avicel, PGU
PF 6	EG I	CMC, β-glucan, xylan, PNP-β-Lac, PNP-β-Cell
PF 7-9	"Traces" of EG I with addition of unknown proteins	Hydrolyzes the same as PF 6 but with lower activities
PF 10-12	Protein mixture with unknown substrate specificity	No measurable activities were detected
PF 13	α-GAL	PNP-α-Gal
PF 14	β-XYL	PNP-β-Xyl, PNP-α-L-Af, xylan
PF 15	XYG	Xyloglucan
PF 16	CBH I	Avicel, PNP-β-Lac, PNP-β-Cell
PF 17	Corresponds to pigmentation yield	No measurable activities were detected

weight substrates as PNP-β-Lac and PNP-β-Cell. Molecular characteristics of the enzyme contained in PF 16 (molecular weight 65 kD, p*I* 4.2) correlate well with same characteristics for CBH I [20]. Hence, PF 6 was identified as EG I and PF 16 as CBH I of *T. reesei*. In the same manner we determined substrate specificity and molecular characteristics of enzymes contained in other PF. Comparison of their properties (Table 1) with current literature data allowed us to make conclusions about component composition of the SA No. 210.29 preparation.

The SA No. 210.29 preparation was found to contain the following enzymes: EG I, EG II, CBH I, CBH II, exo-β-1,3-glucosidase (exo-β-1,3-GL), α-L-arabinofuranosidase (α-L-AF), mannanase (MAN), polygalacturonase (PGA), α-galactosidase (α-GAL), β-xylosidase (β-XYL), and xyloglucanase (XYG) (Table 1, Fig. 1). Moreover, the complex contained other proteins, which did not exhibit significant cellulase, hemicellulase, or pectinase activities, and therefore they were not taken into account during the comparison of component composition of different preparations. It should be mentioned that such known *T. reesei* enzymes as EG III, xylanase I (XYL I), and xylanase II (XYL II) were not found in the SA No. 210.29 preparation.

The suggested technique allowed us not only to perform qualitative analysis of component composition of *T. reesei* enzyme complexes, but also to maintain quantitative estimation of individual enzyme content in the investigated preparations. Estimation of quantitative content of individual enzyme was performed based on chromatofocusing profiles: peak areas corresponding to proteins with 90% purity according to SDS-PAGE data were determined. For enzymes which did not have an individual peak in the chromatofocusing profile (such as α-L-AF, exo-β-1,3-GL, and PGA), we first estimated their content in PF based on their specific activity (towards PNP-α-L-Af, PNP-β-Glu, and PGU, accordingly); after that, knowing the content of corresponding PF in the complex (based on PF peak area) we could calculate their content in the preparation. The results are presented in Table 2. CBH I and CBH II had the highest contents; major enzymes were EG I, EG II, and XYG, while β-XYL, α-L-AF, PGA, MAN, exo-β-1,3-GL, and α-GAL were minor components. As we already mentioned, SA No. 210.29 did not contain EG III, XYL I, and XYL II.

The chromatofocusing profile of *T. reesei* SA No. 210.29 (Fig. 1) supplemented by analysis of activities in fractions (Tables 1 and 2) indeed became a "passport" for this preparation. Development of analogous passports for other enzyme preparations allowed comparing their component composition. Within the scope of this article, we will refer in detail to component composition of laboratory *T. reesei* C-XL No. 592.2 preparation and also examine the composition of commercial preparations Celloviridin G2X and Celloviridin G20X.

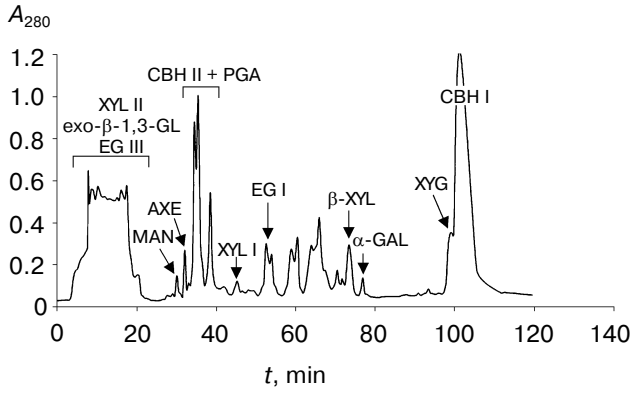

Fig. 4. Chromatofocusing profile for C-XL No. 592.2 *T. reesei* preparation.

Comparison of component composition of *T. reesei* enzyme preparations. The chromatofocusing profile of *T. reesei* C-XL No. 592.2 preparation is presented in Fig. 4. As can be seen, the profile differs from the one belonging to SA No. 210.29 (Fig. 1). In the composition of C-XL No. 592.2 enzyme complex we detected enzymes not present in SA No. 210.29: XYL I, XYL II, EG III, and acetylxylanesterase (AXE). C-XL No. 592.2 preparation compared to SA No. 210.29 exhibited increased content of EG I, β-XYL, exo-β-1,3-GL, also a slight decrease in CBH II and PGA content as well as significant decrease (more than 10-fold) of α-L-AF and EG II.

Quantitative estimations of individual enzyme content in C-XL No. 592.2 and also in commercial preparations Celloviridin G2X and Celloviridin G20X are presented in Table 2. It illustrates that SA No. 210.29 and Celloviridin G2X preparations are very similar in qualitative composition and quantitative content of individual enzymes. Significant difference is exhibited by C-XL No. 592.2, especially in the content of xylanases. Regarding individual enzymes, Celloviridin G20X is quite similar to SA No. 210.29 except for XYL I, XYL II, and EG III, whose content in Celloviridin G20X is higher than in SA No. 210.29, but lower than in C-XL No. 592.2.

Table 2. Quantitative estimation of enzyme content in *T. reesei* preparations

Preparation	EG I	EG II	EG III	CBH I	CBH II	XYL I	XYL II
SA 210.29	++	++	—	+++++	++++	—	—
C-XL 592.2	+++	±	+	+++++	+++	+	+++
Celloviridin G20X	++	++	±	+++++	+++	+	+
Celloviridin G2X	++	++	—	+++++	++++	—	—

Preparation	β-XYL	α-L-AF	PGA	MAN	exo-β-1,3-GL	α-GAL	XYG	AXE
SA 210.29	±	+	+	+	±	±	++	—
C-XL 592.2	+	±	±	±	+	±	++	+
Celloviridin G20X	±	±	+	+	±	±	++	—
Celloviridin G2X	±	+	+	+	±	±	++	—

Note: +++++, enzyme content 30-50%; ++++, enzyme content 20-30%; +++, enzyme content 10-20%; ++, enzyme content 5-10%; +, enzyme content 1-5%; ±, enzyme content less than 1%; –, enzyme is absent.

Table 3. Activities (units/g) of *T. reesei* preparations towards different substrates

Preparation	Protein, mg/g	CMMase	Xylo-gluca-nase	Xyla-nase	Manna-nase	Poly-galactu-ronase	α-Ga-lacto-sidase	β-Glu-cosi-dase	FPA
SA 210.29	278	1400	890	140	130	125	3	14	142
C-XL 592.2	144	1050	570	4720	58	22	1	56	65
Celloviridin G20X	272	1760	1340	1350	114	120	4	30	120
Celloviridin G2X	342	2790	2045	625	85	438	38	45	246

Influence of fermentation conditions on *T. reesei* enzyme biosynthesis. Fermentation conditions have noticeable influences on the composition of secreted *T. reesei* enzymes [23-25]. Preparations of SA No. 210.29 and C-XL No. 592.2 were obtained via cultivation of the same *T. reesei* A1 strain, but under different fermentation conditions. Data characterizing the component composition of the preparations (Table 2) convincingly illustrate that fermentation conditions when SA No. 210.29 was grown resulted in increased biosynthesis of cellulases and virtually suppressed biosynthesis of xylanases. Therefore, we call this type of *T. reesei* fermentation process "cellulose fermentation". Preparation C-XL No. 592.2 was obtained under conditions resulting in increased xylanase biosynthesis, especially XYL II (Table 2), and therefore we call this type of *T. reesei* fermentation process "xylanase fermentation".

Analysis of other preparations obtained under mentioned conditions (data not presented) allowed detecting other characteristic differences in component composition between "cellulase" and "xylanase" *T. reesei* preparations. In general, "cellulase" preparations (compared to "xylanase") exhibit higher content of α-L-AF, MAN, EG II, CBH II, and PGA and also lower content of XYL II, EG III, exo-β-1,3-GL, β-XYL, and AXE. We would especially mention differences in XYL II, α-L-AF, and β-XYL content. Differences in the contents of these enzymes in preparations, which were obtained under "cellulase" and "xylanase" fermentation conditions, can reach 100 and more times (to total absence of these enzymes in the preparation). This fact significantly influences the balance between different activities of enzyme preparations (Table 3). Hence, through variation in fermentation conditions it is possible to obtain *T. reesei* preparations with various component composition and activity towards various substrates.

Finally, it should be noted that 20 commercial and laboratory enzyme preparations based on different *T. reesei* strains were analyzed in this work and differences in their component composition were determined using the proposed technique. Moreover, the correctness of classification for most of the identified *T. reesei* enzymes was confirmed via trypsin hydrolyzate analysis of the corresponding proteins by MALDI-TOF mass-spectrometry (mass-spectra data were processed using MASCOT software: http://www.matrixscience.com and using SWISS-PROT protein database). This part of our work is outside the scope of this article and will be a subject for further publications.

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